University of Alberta

Potential Contribution of 14-3-3α/β to Airway Tissue Remodeling

by

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DEDICATION

To my Father and Mother:
Drs. Houshang Asdaghi and Leila. S. Kouchaki
Again and forever

ABSTRACT

Airway remodeling involves altered epithelial-mesenchymal communication. Our group has recently shown that epithelial cell-derived 14-3-3 proteins, plays a critical role in modulating MMP-1 mRNA expression in fibroblasts.

In this study, Northern and Western-blot analyses were used to evaluate the presence of 14-3-3s in lung epithelial cell-conditioned medium (LCM) and the effect of 14-3-3 in inducing MMP-1 in lung fibroblasts. Response of fibroblasts to 14-3-3 protein in the presence of transforming growth factor-β_1 (TGF-β_1 was studied. My results show that lung epithelial cells (A549) released 14-3-3 proteins. LCM increased expression of MMP-1 dose-dependently, in the lung fibroblast, IMR-90. Immunodepletion of 14-3-3 proteins from LCM decreased its MMP-1-inducing effect. Upregulation of MMP-1 mRNA was sustained following treatment of fibroblasts with TGF-β_1 and LCM.

Thus, lung epithelial cell-derived 14-3-3 may have a potent MMP-1-inducing effect on underlying fibroblasts. This will enhance understanding of the events associated with collagenase production in the lung.

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LIST OF ABBREVIATIONS

α-SMA: Alpha Smooth Muscle Actin

ADA: Adenosine Deaminase

ADAM: A Disintegrin and Metalloproteinase

AHR: Airway Hyperresponsiveness

ASM: Airway Smooth Muscle

BAL: Bronchoalveolar Lavage

BHR: Bronchial Hyperresponsiveness

CSF: Cerebrospinal Fluid

DCs: Dendritic cells

DMEM: Dulbecco's Modified Eagle's Medium

ECM: Extracellular Matrix

EMTU: Epithelial Mesenchymal Trophic Unit

EGF: Epidermal Growth Factor

EGFR: Epidermal Growth Factor Receptor

ET: Endothelin

FBS: Fetal Bovine Serum

FGF: Fibroblast Growth Factor

GPCRs: G-Protein-Couples Receptors

IGF: Insulin- like Growth Factor

IGF-BP: Insulin-like Growth Factor – Binding Protein

KCM: Keratinocyte Conditioned Medium

KDAF: Keratinocyte-Derived Anti-fibrogenic Factor

LCM: Lung Epithelial-Cell Conditioned Medium

LDH: Lactate Dehydrogenase

MAPK: Mitogen Activatated Protein Kinase

MCP: Monocyte Chemotactic Protein

MMP: Matrix Metalloproteinase

MMP-1 : Matrix Metalloproteinase-1, Collagenase

mRNA: Messenger Ribonucleic Acid

NF-kB: Nuclear Factor- kappa B

PDGF : Platelet Derived Growth Factor

PTPH : Protein-tyrosine Phosphatase

SDS-PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

Ser: Serine

STAT: Signal Transducer and Activator of Transcription

Shh: Sonic Hedgehog

Tg: Transgenic mice

TGF-β: Transforming Growth Factor-beta

TH cells: T helper cells

Thr: Threonine

TIMP:Tissue Inhibitors of Metalloproteinase

TNF: Tumor Necrosis Factor

On Asthma by Maimonides

....I know from what I have witnessed with my own eyes and from what my Master has described to me that the cause of this asthma [from which he suffers] is a defluxion that descends from the brain at certain times [of the year], but mostly in winter. [And I know] that the orthopnea and distress do not cease through the night for days on end, according to the length or brevity of the attack, until the defluxion decreases and until that [fluid] which has reached the lungs has been cocted so that the latter have become clean. This is what I know about the cause of this disease.

....You [my patient], have also informed me that from sheer necessity you resort to purging once or twice a year, with that whose property is to expel the phlegm and cleanse the brain and lungs. And [I know] that you often take a purgative in the case of an attack and [by] that [means] you get rid of it. I also know that you are middle-aged and that your body is intermediate between leanness and heaviness and that your general temperament of your brain is very close to the balanced type, though it tends somewhat toward heat, and that the temperament of your brain is hotter than it should be. I inferred this from the fact that you are harmed- as you told me- by the odors of hot ingredients, that you can not stand their smell, that your hair feels very heavy for you, that you find relief [only] by shaving it very frequently, and that you are [made] very uncomfortable by covering your head with a turban. All these [symptoms] indicate [excessive] heat of [your] brain....

Maimonides (1135-1204), Doctor and Philosopher

Adopted from, On Asthma – by Moses Maimonide

Translated and annotated by Gerrit Bos, 2001

Chapter 1

Review of literature

1.1 Introduction

Asthma is a serious public health problem that affects people of all ages, races, and ethnicities. The World Health Organization has estimated that between 100 million and 150 million people worldwide (up to 12% of the population) suffer from asthma. In the past 20 years the prevalence of asthma has significantly increased in both developed and developing countries, and this trend appears to be particularly marked in children, making asthma the leading cause of hospitalization among young children. The epidemic increase in asthma has been attributed to aspects of Western culture, including outdoor and indoor air pollution, childhood immunization, and cleaner living conditions but no single cause has been identified. And despite significant advances in our understanding of the disease and availability of more effective therapies, asthma remains a heavy burden on health-care systems.

Asthma has been the focus of media, public health, and research initiatives to improve awareness and compliance with medications and to understand the causes and course of disease. The first step in developing improved therapeutic modalities for treating chronic respiratory diseases like asthma is to understand the primary cellular and genetic mechanisms and triggers that ultimately result in a particular disease phenotype.

1.2 Historical Perspective of Asthma

Henry Hide Salter in 1859 was the first to describe asthma as a disease of reversible airway obstruction[1]. In 1960 after a century of research the definition of asthma still remained the same with the inclusion of bronchial hyperresponsiveness (BHR).

Although the presence of eosinophilic infiltration in the airways has been known for almost 100 years, having been first described in 1908 in a patient who died of asthma[2].Yet, inflammation was not believed to be a cause of asthma until recently. In the early 1980s, the development of flexible, fiberoptic bronchoscopy permitted evaluation of the airways in groups of asthmatics with well-defined lung function during periods of disease inactivity. These studies revealed that even when patients were asymptomatic, inflammation of the airways was present. Around the same time, the seminal papers describing CD4 Th subsets and their functional effects were published[3,4]. These findings, together with the association of Th2 cytokines and allergic diseases, led to the theory that Th2 cells promote asthma, and therefore for about 15 years asthma research has almost exclusively focused on inflammation as a cause of disease. By 1997, the National Hearth, Lung, and Blood Institute defined asthma as "a chronic inflammatory disorder of the airways in which many cells play a role, in particular mast cells, eosinophils, and T lymphocytes. In susceptible individuals this inflammation causes recurrent episodes of wheezing, breathlessness, chest tightness, and cough particularly at night and/or in the early morning. These symptoms are usually

associated with widespread but variable airflow limitation that is at least partially reversible, either spontaneously or with treatment. The inflammation also causes an associated increase in airway responsiveness to a variety of stimuli[5].

Over the last 30 years, this expansion of the definition of asthma illustrates the rapid progress that has been made in understanding this complex disease. This increase is due in part to the progress made in the techniques used to investigate the disease. Originally, investigations into asthma were restricted to the measurement of airways obstruction and inflammatory cell numbers in the peripheral blood, whereas much of the early pathologic investigation relied on the use of postmortem samples. It was not until the advent of the fiberoptic bronchoscope that the detailed pathology of asthma was advanced in a systematic manner. Fiberoptic bronchoscopy is now a commonly used technique in the field of respiratory disease research [6,7] allowing collection of biopsy specimens, bronchial brushing samples, and bronchoalveolar lavage (BAL) fluid for immunochemical or molecular analysis and, more recently, for establishment of in vitro models, enabling functional studies aimed at defining underlying disease mechanisms.

1.3 Asthma a disease of inflammation and remodeling

Evidence that inflammation was a component of asthma was initially derived from findings at autopsy in patients with fatal asthma. Their airways showed infiltration by neutrophils, eosinophils and degranulated mast cells[8]. These findings were therefore considered to be characteristic of fatal asthma, but not

necessarily of other forms of the disease. Today immunohistochemical analysis of bronchial biopsy specimens has enabled detailed description of the inflammatory cells present in asthmatic airways. These infiltrates are characterized by the presence of mast cells, basophils, eosinophils, monocytes, and T lymphocytes of a Th_2 inflammatory profile[9]. Both acute and chronic inflammation is orchestrated, in part, by various T cell- and mast cell-derived cytokines, such as interleukin (IL)-1β, IL-5, IL-4, IL-9, IL-10, IL-13 and tumour necrosis factor (TNF), and mediators such as the cysteinyl leukotrienes and isoprostanes, that are increased in asthmatic airways[10]. Consequently, treatments have been developed to suppress this inflammatory response for therapeutic benefit[10]. Some success has been achieved with the use of corticosteroids, leukotriene receptor antagonists, and mast cell stabilizers[11]. However, there are many asthmatic patients with chronic symptoms for whom anti-inflammatory therapy is inadequate and who account for up to 50% of the health costs of this disease[12].

Although inflammation undoubtedly plays a central role in asthma, inflammation alone does not explain many of the features characteristic of this chronic and relapsing disease[13]. Eosinophils have been assumed to play a central role in disease pathogenesis. However, studies with recombinant human IL-12[14] or an anti–IL-5 blocking mAb[15,16] have failed to reveal efficacy, despite markedly reducing circulating and sputum, but not tissue eosinophils. Thus, despite being associated with asthma atopy and airway eosinophilia would not seem to be critical requirements for disease expression. Genetic studies have also

demonstrated that atopy and BHR have different patterns of inheritance[17]. These findings collectively imply that locally operating factors such as genetics and environmental factors, play an important role in predisposing individuals to asthma[18]. This may provide an explanation for the epidemiologic evidence that identifies environmental factors, such as pollutant exposure[19,20], diet[21] and respiratory virus infection[22], as important disease risk factors.

Structural changes of the airway wall, collectively referred to as airway remodeling, may be a result of either the interaction of inflammatory mediators with stromal cells or tissue injury[23]. Local factors, including the structural cells in the airway and the matrix, respond to inflammation in a characteristic, coordinated fashion that may be an attempt to repair the damage caused by local inflammation — an effort to keep the airway intact.

The traditional viewpoint that inflammation is the cause of asthma has meant that airway remodeling, the other major histologic feature of the disease, has received considerably less attention, and its relevance to disease pathogenesis is still controversial[24]. Yet, in addition to inflammatory cells, changes involving epithelial goblet cell hyperplasia and metaplasia, collagen deposition and thickening of the lamina reticularis, smooth muscle hyperplasia, and proliferation of airway blood vessels and nerves are consistently observed in ongoing disease, as well as in postmortem asthmatic airways (Figure 1.1)[25,26]. Conversely studies of the endobronchial biopsies of children with severe asthma showed opposing results[27]. It is now known that in spite of long-term corticosteroid treatment, airway remodeling remains a feature of asthma. This may undermine the

paradigm that airway inflammation is solely responsible for the manifestation of asthma and that airway remodeling may occur in parallel or even independently from inflammation.

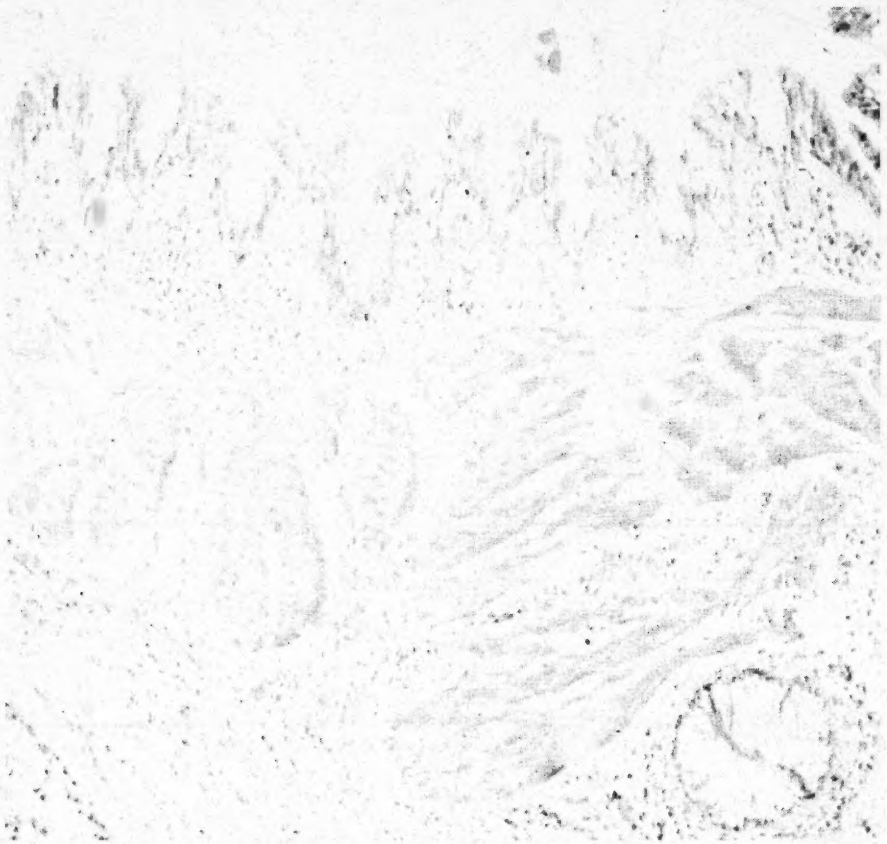


Figure 1.1: Histologic section through an asthmatic airway. The plate shows that, in addition to the presence of large numbers of inflammatory cells, there are characteristic structural changes, including epithelial goblet cell hyperplasia and metaplasia, thickening of the lamina reticularis, and increased amounts of smooth muscle.

Davies DE et al., J Allergy Clin Immunol. 2003 Feb;111(2):215-25

1.4 Components of Airway remodeling

Much research has focused on the pathophysiologic changes associated with asthma and on the contribution of individual structural components to the process of airway remodeling (Figure 1.2)[28]. The following discussion will explore the physiologic consequences of the individual abnormalities comprising airway remodeling and will correlate structural changes with physiologic measurements of lung function.

1.4.1 Airway Epithelial Injury

Human bronchial epithelial cells play an important role in maintaining the structural and functional integrity of the lung. It provides a barrier between the tissue and the airway lumen. The epithelial layer regulates several key physiological functions of the airways, including fluid and ion transport into airway lumen and mucociliary clearance[29]. It is actively engaged in communicating with cells of the immune and inflammatory systems, as well as secreting cytoprotective molecules[30].

In asthma, the airway epithelium is abnormal. Mast cells, dendritic cells (DCs), lymphocytes, and eosinophils are readily identified abutting the basal cell layer. Epithelial desquamation[31,32], although somewhat controversial[33] remains a characteristic feature of the disease. Epithelial shedding is not observed in other lung inflammatory diseases, such as chronic obstructive pulmonary disease, in which the epithelium becomes multilayered because of squamous metaplasia.

Desquamation appears to be due to apoptotic cell loss[33] and is accompanied by increased expression of the epidermal growth factor receptor (EGFR) in areas of damage, which is consistent with damage occurring in vivo[34]. However, the increase in epithelial EGFR is paradoxical because, unlike chronic obstructive pulmonary disease, there is little evidence of increased proliferation[35,36]. This might be due to high expression of the cyclin/cyclin-dependent kinase inhibitor $p21^{waf}$, particularly at the severe end of the disease spectrum[35]. This negative regulator of G1 cyclins can be induced by the antiproliferative transforming growth factor TGF-β_1 and β_2, the levels of which are increased in patients with asthma [35,37,38]. As a consequence of $p21^{waf}$ expression, the proinflammatory phenotype of the epithelium can be maintained by prolonging the period of epithelial repair. In addition, the EGFR might contribute directly to these proinflammatory responses because epidermal growth factor is known to elicit IL-8 expression and release from bronchial epithelial cells, a response that is not fully suppressed by corticosteroids[24]. Because the EGFR is overexpressed in the morphologically intact epithelium of patients with severe asthma and strongly correlates with epithelial IL-8 expression[39], this might contribute to airway neutrophilia in patients with severe asthma.

Taken together, evidence suggests that epithelial cell damage may reflect the severity of illness, although contemporary thinking also emphasizes that fundamental alterations in epithelial phenotype may promote airway smooth muscle hyperreactivity or stimulate mesenchymal cell migration and matrix deposition[40]. This suggests that epithelial damage results in aberrant signalling

between damaged cells and the underlying mesenchymal layer. The cross talk between epithelial and mesenchymal cells (e.g., airway smooth muscle, fibroblasts, myofibroblasts) is termed the epithelial-mesenchymal unit (EMTU, see below).

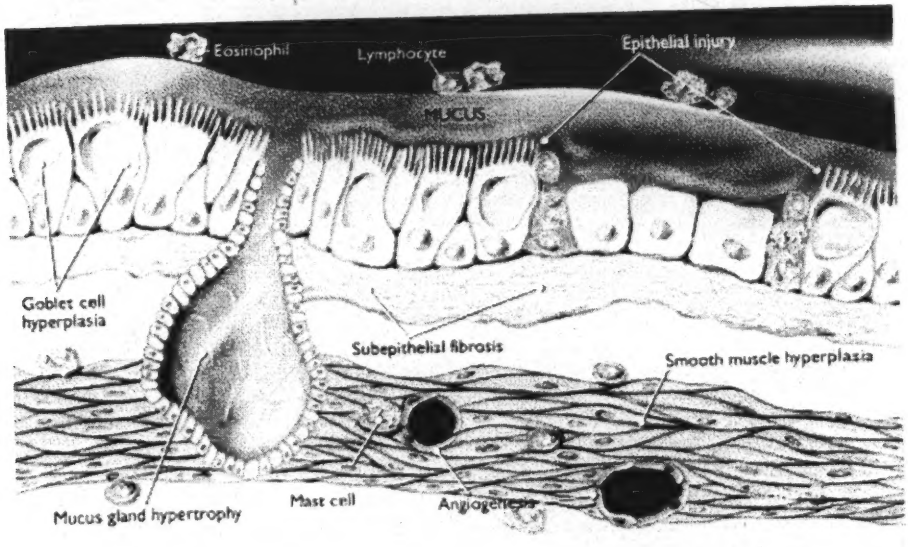


Figure 1.2: Integrative model of the components of airway remodeling. In asthma patients, airway inflammation, epithelial and goblet cell phenotype alterations, subepithelial fibrosis, excess mucus secretion, smooth muscle cell hypertrophy and hyperplasia, and angiogenesis are present. *Copyright 2003, Ethan F. Geehr.*

1.4.2 Thickening of lamina reticularis

Ultrastructural and immunohistologic studies of bronchial biopsy specimens obtained from subjects with asthma show that the apparent basement membrane thickening, defined as subepithelial fibrosis, is caused by the deposition of fibrillar collagen, which leads to an increase in the depth and density of the lamina reticularis[33]. In normal subjects lamina reticularis, produced by mesenchymal-derived cells[41,42], is composed of laminin and type IV collagen, to which epithelial cells are attached. The subepithelial fibrosis seen in asthmatic subjects is mainly caused by the abnormal deposition of type I and III collagens and fibronectin[43]. Increases in the lamina reticularis, however, also occur in patients with allergic rhinitis who have a normal FEV_1; thus, this finding is not unique to asthma[44]. Furthermore, thickening does not correlate with the duration or severity of asthma, as a substantial increase in the lamina reticularis is observed in children and patients with mild asthma[45-48]. In contrast, many others report a direct correlation between subepithelial fibrosis and asthma severity and an inverse correlation with FEV_1 [49-51]. The disparity among these studies may be attributable to several factors, including differences in the use of inhaled corticosteroids. Hence, lamina reticularis thickening may not be sensitive for predicting asthma severity[45,47] and is unlikely the sole predictor in patients who develop irreversible airflow obstruction.

Evidence suggests that deposition of collagen extends into the submucosa in patients with asthma[52]. Functionally, increased collagen deposition surrounding

airway smooth muscle may increase wall stiffness, somewhat analogous to scar formation[53]. In fact, measurements of airway distensibility are abnormal in wheezy infants and in patients with mild asthma[54,55]. Attenuated airway wall compliance due to fibrosis occurs in patients who are clinically asymptomatic compared with healthy subjects[54]. When airway distensibility, rather than FEV_1, is used as a measure of remodeling, there is an inverse relation between distensibility and subepithelial fibrosis[56].

Other abnormalities of airway matrix deposition in asthma include an increase in expression of tenascin and specific isoforms of laminin that are associated with tissue injury[57]. Moreover, proteoglycans such as hyaluronan, versican, and decorin, are overexpressed in the asthmatic airways[58]. In addition, studies suggest that BHR correlates with the degree of proteoglycan deposition both in vitro[59] and in vivo [58].

The physiological relevance of alterations in matrix deposition in the bronchial wall of asthmatic patients is still unclear. The deposition of proteoglycans and glycosaminoglycans likely contributes to the altered mechanical properties of the airway wall and may promote airway obstruction. Other important aspects of remodeling include degenerative changes in bronchial cartilage, along with loss of elastic recoil and fragmentation or loss of elastin fibrils[60-62]. Clearly, further studies aimed at defining the functional relation between matrix alterations and increased airway obstruction is warranted.

1.4.3 Mucus gland hypertrophy and hyperplasia

In contrast to the conflicting data regarding lamina reticularis thickening, a consistent association between asthma and mucus gland hypertrophy and hyperplasia has been shown. Neither the distribution of mucus expression nor the degree of goblet cell hyperplasia, however, correlates with asthma severity[63,64]. Moderate asthma may be associated with more secreted mucin as compared with in mild asthma, whereas fatal asthma may be associated with both increased mucus gland and goblet cell area that increases airway mucus quantity as compared with in nonfatal asthma[63]. Studies aimed at understanding mucus secretion in asthma may offer new therapeutic targets in this disease.

1.4.4 Airway smooth muscle hypertrophy and hyperplasia

Increased airway smooth muscle (ASM) mass, which is attributed to increases in both myocyte number (hyperplasia) and size (hypertrophy), is a well-documented pathologic finding in the airways of patients with chronic severe asthma[65,66](Figure 1.3). Traditional views assert that airway smooth muscle is a structural effector tissue in the bronchi that solely regulates airway caliber and bronchomotor tone[67]. The ASM contraction is achieved through stimulation of G-protein-couples receptors (GPCRs) by contractile agonists such as histamine, leukotriene C_4 and acetylcholine[67]. Today accumulating evidence suggest that ASM cells are multifunctional, having the capacity for contraction, migration, proliferation, and synthesis of ECM, growth factors, cytokines, and chemokines[68,69].

Figure 1.3: Airway smooth muscle hypertrophy and hyperplasia. Histologic section through an asthmatic airway shows considerable hyperplasia and hypertrophy of smooth muscle (black arrow) and pronounced thickening of the basal membrane (hashed arrow). Biopsy was obtained from a patient who died of status asthmaticus.

Bronchial Hyperresponsiveness, Philip H. Quanjer et al. Copy right 2005

Therefore, ASM may become an active participant in the pathogenesis of chronic inflammatory airway diseases since different pro-inflammatory cytokines[70] as well as different mitogens can induce profound changes in the intrinsic properties of ASM[71,72]. Further, as a population, airway myocytes display marked phenotypic heterogeneity based on expression of genes encoding contractile proteins, ECM, receptors, and cytokines and appear to be comprised of divergent mesenchymal sublineages[69,73,74]. In this regard exogenous stimuli, differentially effect myocyte responses in a phenotype-dependent manner [74,75]. Collectively, this ability for functional plasticity uniquely equips bronchial myocytes to differentially regulate airway lumen diameter both transiently, via reversible contraction, and chronically, via structural remodeling due to fibrosis and muscle hypertrophy[76].

1.4.5 Mucosal neovascularization

Compelling evidence suggests that angiogenesis occurs in chronic asthma[77,78], although some argue that it is the size, not the number, of blood vessels that is increased[79,80]. Not surprisingly, airway mucosal blood flow also is increased[81]. In asthma, changes in the microvasculature of airways is out of proportion to the increased metabolic needs of tissues and it is thought to be due to overproduction of growth factors that stimulate vessel growth and remodeling. Also, blood vessels in diseased tissues usually have multiple abnormalities, ranging from the expression of molecules not found on normal vessels to alterations in endothelial barrier function and leakiness[82]. In fact, current data support a correlation

between the number of blood vessels in the bronchial wall and the severity of asthma[77,83]. Interestingly, increased subepithelial microvessels in the tracheal mucosa were found even in steroid-naive patients with newly diagnosed asthma, suggesting increased airway vascularity may be completed when asthma symptoms begin[84].

In short, many studies report alterations in the structural components of the bronchi in patients with asthma; however, the clinical relevance of such histologic changes remains unclear[28]. Some of these alterations, such as mucus gland hypertrophy and mucosal neovascularization, are associated with severity of asthma. In contrast, a consensus is forming that lamina reticularis thickening may not be specific for asthma and may not correlate with disease severity[28]. A direct correlation between any one component of airway remodeling and the severity of asthma, however, remains tenuous.

1.5 Effector pathways that enhance asthma

1.5.1 Th2 Cytokines

$CD4^+$ Th2 lymphocytes contribute to the inflammatory response and to airway remodeling by producing cytokines. Each cytokine has distinct functional effects in induction of disease, but IL-13 predominates in its contribution to the pathophysiology in asthma[85]. CD4 lymphocytes produce a majority of the Th2 cytokines in the respiratory tract in asthma. In airway biopsies and BAL cells from asthmatics, IL-4, IL-5, and IL-13 colocalize with T-cell markers in a

majority of, but not all, cells[86]. Yet, CD8, γ/δ and NKT cells, eosinophils, mast cells, basophils, NK cells, and subsets of Class II MHC-expressing accessory cells can produce Th2 cytokines[86-90]. IL-4, IL-5, and IL-13 produced by non-CD4 T cells may be essential for the development and perpetuation of asthma. The period of cytokine secretion from this array of cells will vary. Thus, as lymphocytes die or cease production of cytokines after their activation, waves of cytokines produced by non-CD4 T cells may follow. For example, IL-4 production activates eosinophils, and eosinophils then produce IL-4[91]. Th2 cell production of IL-25 also enhances cytokine production[87]. IL-25 is a cytokine from the IL-17 family that is produced by Th2 cells and mast cells and promotes Th2-induced lung pathology[87,92]. Taken together, these studies highlight the significance of effector cytokines produced by nonlymphocytes. In asthmatics, enhancing Th2 cytokines will increase the magnitude of and/or prolong the immune response.

1.5.2 Eosinophilia

Airway eosinophilia is the most characteristic finding in asthma and has been considered central in the pathogenesis of disease. Yet, to date, researchers have not established a precise role of eosinophils in disease development and persistence. Animal studies have detailed the pathways by which eosinophils are mobilized from the bone marrow and recruited to the respiratory tract. These studies have also defined how individual Th2 cytokines control these steps. Airway eosinophilia depends on both IL-5 and STAT-6 signalling[91]. In the

absence of IL-5, blood and BAL eosinophils are not increased in response to Th2 cell activation, yet eosinophils are present in the airway tissue at approximately one third the level observed in IL-$5^{+/+}$ mice[91,93-95]. In mice lacking effective signalling for IL-4 and IL-13, few eosinophils were observed in the airway tissue or in BAL in response to Th2 cell activation in the respiratory tract. This occurred despite the ability of these mice to generate and release eosinophils from the bone marrow[96,97]. Associated with these findings was a marked reduction in eosinophil-recruiting chemokines including eotaxin-1. Thus, CD4 T cells provide essential signals for eosinophil mobilization, activation, and recruitment to the respiratory tract. There are also other strain-specific factors in mice that lead to different levels of eosinophils in the respiratory tract in response to comparable Th2 stimuli[98].

Once eosinophils have been recruited to the respiratory tract, their activation leads to secretion of major basic protein, eosinophil cationic protein and eosinophil peroxidase; cytokines such as TNF, GM-CSF, IL-4, IL-13, and IL-5; chemokines including RANTES and eotaxin; and platelet-derived growth factor[91]. These factors damage airway epithelial cells, stimulate mucus secretion and fibrosis, and induce bronchospasm and BHR. Phipps *et al*[99] using an allergen-induced cutaneous model of asthmatic inflammation, showed that eosinophils can influence airway function through tenascin production and the release of TGF-β .

The role of eosinophils may depend on the clinical severity of asthma[100,101]. Traditionally, mast cells are thought to be responsible for the acute phase of the

asthmatic response through IgE-mediated histamine release and smooth muscle stimulation. By definition, mild asthma can include BHR and acute periods of bronchospasm, often allergy-related[102]. However, mild asthma is generally characterized by near normal lung function and the absence of frequent exacerbations requiring hospitalization. Thus, in a recent study, it was noted that, in moderate to severe asthmatics, management of asthma on the basis of sputum eosinophil profiles resulted in improved symptoms and outcomes compared with treatment according to clinically defined standard protocols[103].

1.5.3 Adenosine

Adenosine is generated by dephosphorylation of adenine nucleotides released from inflammatory and injured cells. In asthma, adenosine is increased in the bloodstream and airways[104,105]. Adenosine has long been known to induce bronchoconstriction in asthmatics, but not in normal subjects. It can engage cell-surface adenosine G-protein-coupled receptors on mast cells, eosinophils, macrophages, neurons, epithelial cells, and smooth muscle cells. Adenosine can be removed by metabolism by adenosine deaminase (ADA) or adenosine kinase. Recent studies in CC10 IL-13 transgenic (Tg) mice show that IL-13 induced high levels of adenosine and decreased ADA activity[106]. When ADA was administered to IL-13 Tg mice and adenosine levels were returned close to normal, airway inflammation was reduced and there was minimal subepithelial fibrosis. Parallel to these findings, ADA-deficient mice had high levels of adenosine and a lung phenotype strikingly similar to CC10 IL-13 Tg mice with

high levels of IL-13. This pathology was largely blocked with an IL-13 inhibitor. Thus, adenosine stimulates IL-13, and IL-13 stimulates adenosine. This link is critical for expression of allergic airway inflammation and remodeling in mice. In asthma, adenosine is likely generated from inflammatory cells, damaged airway epithelium, and other structural cells in the lung[107]. IL-13 produced in the lung may inhibit ADA, which increases adenosine levels and stimulates release of more IL-13. This in turn activates effector pathways that damage tissue, leading to the generation of more adenosine. In these studies, blocking the increase in adenosine by administering ADA relieved the cycle, which suggests that adenosine stimulates a nonredundant pathway to increase IL-13 and promote disease[106].

1.5.4 Monocyte chemotactic protein-1

Monocyte chemotactic protein-1 (MCP-1) also appears to be involved in a chain of inflammatory events that promotes airway remodeling in asthma. MCP-1 is elevated in the airways of asthmatics[108]. MCP-1 is produced by bronchial epithelial cells, macrophages, and smooth muscle cells[108]. In mice, IL-13-induced inflammation is associated with an increase in MCP-1 release[109]. Recent studies indicate that adenosine, through its effects on the A2B receptor, increases the release of MCP-1 from bronchial smooth muscle cells[110]. This may be one pathway by which IL-13 increases MCP-1. MCP-1 may control multiple different aspects of the Th2 inflammatory response. First, it attracts effector/memory Th cells[111]. Therefore, circulating Th2 cells will be recruited to

the airways. MCP-1 has been shown, in some studies, to enhance Th2 cell differentiation. Naive CD4 T cells stimulated with antigen and MCP-1 induced IL-4 but not IFN-γ [112], and MCP-1-deficient mice could not be induced to generate Th2 cells[113]. The mechanism by which MCP-1 influences Th2 cell generation has not been defined. In other studies, a deficiency in MCP-1 did not polarize strictly along Th1/Th2 lines[114], and mice with a deficiency in CCR2, the primary receptor for MCP-1, exhibited enhanced eosinophilia and a defect in IFN-γ production[115], suggesting that MCP-1 effects may not be Th2 cell specific. MCP-1 stimulates mast-cell mediator release[116], including Th2 cytokines, which will promote further Th2 cell generation. MCP-1 also acts to induce remodeling of the airways by stimulating TGF-β1 release from macrophages and fibroblasts[117,118] and by promoting airway fibrosis[119]. The induction of MCP-1 in a Th2 inflammatory response can amplify Th2-induced effects in a number of ways and promote subepithelial fibrosis. Upon stimulation with MCP-1, fibroblasts produce more MCP-1, which recruits Th2 cells, which in turn produce more IL-13, which stimulates more MCP-1 release, and the cycle continues.

1.5.5 Matrix metalloproteinases (MMPs)

MMPs are endopeptidases belonging to the metzincin superfamily. Members of this large superfamily depend on the presence of a zinc ion for their catalytic activity (hence the name "metallo" proteinase)[120]. The MMP family contains at least 26 known members, which are grouped according to their substrate specificity. The collagenases (MMP-1, -8, and -13) degrade fibrillar forms of

interstitial collagen. The gelatinases (MMP-2 and -9) are specific for denatured collagens and collagen-IV of the basement membrane. Stromelysins (MMP-3, -10, and -11) primarily cleave noncollagen components of ECM such as fibronectin (FN), laminin (LN), and vitronectin (VN). MT-MMPs (MMP-14, -15, -16, -17, and -24) are membrane-type MMPs found on the surface of many cell types. The final subgroup of MMPs referred to as "other" includes matrilysins (MMP-7 and -26), metalloelastase (MMP-12), enamelysin (MMP-20), and other MMPs with less defined characteristics[120]. In addition to MMPs, the metzincin superfamily also contains cell surface transmembrane proteins known as a disintegrin and metalloproteinase (ADAMs). Although ADAMs posses metalloproteinase-like activity, they are distinguished from MT-MMPs by the presence of two functional domains: a disintegrin-like domain that is structurally similar to snake venom disintegrins. This domain promotes adhesion of the molecule to cell-surface integrins. A metalloproteinase domain that can cleave cell-surface cytokines into their active forms, release receptors, and act on numerous other cell-surface molecules[121]. There are currently more than 30 known cell-surface ADAMs. In addition, there is a growing number of soluble ADAMs that have been designated ADAMTS due to the presence of thrombospondin-type repeats[122].

MMPs are responsible for extracellular matrix remodeling during normal physiologic events, like embryogenesis and wound repair. Their ability to break down collagen and elastin, the major structural proteins in the lung, imply that they play a major role in the development of lung pathology. Yet, MMPs also

have essential roles in leukocyte migration from the vascular space to sites of inflammation and cleavage of molecules, such as adhesion receptors and membrane-bound and inactive cytokines[123].

MMPs modulate inflammation and remodeling in different models of asthma. MMP-1, -2, -3, -9, and MMP -12 are all elevated in asthmatics, although MMP-9 is the predominant MMP in asthma[124,125]. MMP-9 is consistently increased in blood, BAL fluid, sputum, and biopsies of asthmatics, and it is increased in the BAL fluid 24 hr after antigen challenge[126,127]. Tissue inhibitors of metalloproteinase (TIMPs) are the specific inhibitors of MMPs, and TIMP-1, which covalently binds MMP-9, is also increased in asthma. During acute asthma exacerbations, the ratio of MMP-9 to TIMP-1 increases, and this has been hypothesized to promote airway remodeling[128]. Studies with MMP-12 deficient mice bred to CC10 IL-13 Tg mice show a reduction in airway fibrosis as well as a lower eosinophil cell count in BAL. This implicates that MMP-12 may play a role in eosinophil recruitment to the respiratory tract and subepithelial fibrosis in this asthma model[129]. Elevation in MMP-2 activity in mice with allergic airway inflammation regulates inflammatory cell migration within the lung[130]. When MMP-2 effects are blocked, antigen-induced inflammation, including lymphocytes and eosinophils, is markedly reduced in the airway lumen (BAL), but enhanced in the lung parenchyma and around the airways, indicating a defect in migration out of the airway tissue. These findings are associated with reduced eotaxin levels in the BAL, suggesting that MMP-2 modulates leukocyte chemoattractants in asthma. The potential mechanisms of MMP-2 activity

include acting directly or indirectly on the epithelium to release chemokines, or degrading of chemokines in the airway tissue to generate a gradient[131,132].

MMP-1 is mainly secreted by fibroblasts and activated monocytes/macrophages[133,134]. Although MMP-1 displays a rather limited proteolytic spectrum in the context of extracellular matrix, it is a major fibrillar collagen degrading protease[135]. It is, therefore likely that in asthmatic subjects, increased levels of MMP-1 is mainly associated with bronchial remodeling involving impaired cycles of degradation and repair. In addition, it is important to notice other catalytic activities of MMP-1 in the asthmatic airways. One of which is its contribution to the insulin-like growth factor (IGF) axis[136]. Evidence have shown that IGFs and IGF-binding proteins (IGF-BPs) are important regulators of airway smooth muscle (ASM) cells growth and proliferations[137]. The bioavailability of IGFs to exert its proliferative activity on smooth muscles depends on the proteases that could cleave the high affinity -inhibitory IGFBPs[138,139]. Rajah et al have shown, the expression of immunoreactive MMP-1 to be dramatically increased in airway smooth-muscle cells of asthmatics, as quantified using both visual and densitometric analyses[140]. Further, it was demonstrated that MMP-1 functions as an important protease to degrade insulin-like growth factor binding proteins (IGF-BP)-2 and -3, thus increasing the availability of IGF-1, a potent growth factor for airway smooth muscles[125]. Taken together the above observations provide strong evidence for the implication of IGFBP proteolysis by MMP-1 in the pathophysiology of asthmatic airway remodeling.

1.6 Epithelial-mesenchymal trophic unit (EMTU)

The importance of epithelial-mesenchymal interactions in different tissues of the body has been known for many decades[141-145].

In lung this communication is vital for normal morphogenesis during embryonic development[146]. In humans, branching morphogenesis of the 23 airway generations is normally complete by week 25 of gestation. There is some overlap with the formation of alveoli, which normally commences at week 20 and continues for several years postnatally[147]. Seminal experiments conducted by Alescio, Colombo and Piperno[148] showed that when distal lung mesenchyme was engrafted onto tracheal epithelial strips the epithelium began to branch and reverted to an alveolar epithelial cell phenotype. By contrast, if lung epithelium was engrafted onto tracheal mesenchyme, the epithelium was re-programmed to display a tracheal phenotype[148]. From these experiments, it was suggested that instructive signalling was unidirectional; the pulmonary mesenchyme, via production of growth factors and other signalling molecules such as nuclear factor- κB[149], TGF-β3 and fibroblast growth factor (FGF)-7 and -10[150], was absolutely required for normal epithelial development. However, it is now known that the epithelium also produces signalling molecules such as EGF[151], TGF-β2[152], Sonic Hedgehog (Shh)[153]and Wnt[154], proteins that are important for normal mesenchymal differentiation and proliferation. Therefore lung epithelial cells and fibroblasts are important sources of growth factors, transcriptional factors, signalling molecules and cytokines by which means they communicate amongst

themselves and other cells of the body. This bidirectional cross talk is pivotal in modelling of the airways and development of lung parenchyma during embryogenesis. Today there is accumulating evidence to suggest that molecular pathways responsible for pathological airway remodeling is similar to interactions that lead to branching morphogenesis, "modeling", of airways. Therefore, the next part of this chapter will focus on examples of how the mechanisms of lung development can be recapitulated in airway remodelling in asthma.

1.7 Airway remodeling in asthma and EMTU

Although asthma is a heritable trait, the increasing prevalence of this disorder over the last 30 years is too large and widespread to be caused by genetic change. This has led to the proposal that the increasing trends in asthma are due to changes in the environment acting on a susceptible genotype both in respect to disease induction and worsening of established disease[18]. This proposal is supported by epidemiologic studies identifying multiple interacting environmental risk factors for asthma induction and exacerbations [155,156]. As the barrier to the external environment, the bronchial epithelium is in a key position to translate gene-environment interactions. The bronchial epithelium is actively engaged in defence of the airways by secreting mucus and cytoprotective molecules that trap and inactivate inhaled substances which are then removed through ciliary beat activity. It also responds to environmental stimuli by signalling to, and interacting with, cells of the innate and adaptive immune

systems through secretion of cytokines and chemokines and expression of adhesion molecules such as ICAM-1 and CD40[157]. These interactions enable the epithelium to work in conjunction with the immune system to provide a mechanism whereby extra protection can be recruited when the natural epithelial barrier is compromised and, once recruited, the immune and inflammatory cells can themselves promote tissue repair by removing cell debris and providing a transient supply of locally acting growth factors. As discussed earlier in this chapter, evidence is now accumulating that the epithelium of a patient with asthma is structurally and functionally abnormal[31,158]. Increased epithelial "stress" is often observed in the guise of increased expression of proinflammatory transcription factors such as NFκB, activator protein-1[159], signal transducer and activator of transcription (STAT)-1[160]and STAT-6, the signal transducer utilized by interleukin-4 and interleukin-13[161]. In addition as described before (section 1.3.1), expression of EGFRs is markedly upregulated in the epithelium of adult asthmatics[34]. This was a paradoxical finding, in that epidermal growth factor analogs stimulates epithelial cell migration, proliferation and differentiation, and EGFR upregulation does not correlate with the proliferative status of the repairing epithelium in asthma. Moreover, levels of TGF-β remain elevated in induced sputum from asthmatics despite corticosteroid therapy effectively reducing the presence of inflammatory cells[162](section 1.3.1).

How does this relate to re-activation of the EMTU? In addition to being more susceptible to damage, normal repair processes are also compromised. Failure of appropriate growth and differentiation of airway epithelial cells will cause

persistent mucosal injury, locking the airway into a cycle of aberrant repair[163]. In extreme cases in which the basement membrane is damaged, epithelial cells and fibroblasts might directly interact with each other. Restitution of damaged epithelium is associated with alterations in cell phenotype, as well as disruption of the normal pattern of proliferation and apoptosis. These processes are spatially and temporally controlled by local signals generated by a plethora of growth factors and the extracellular matrix[164]. Injury to epithelial monolayers results in increased release of fibroproliferative and profibrogenic growth factors including fibroblast growth factor (FGF-2), insulin growth factor (IGF-1), platelet derived growth factor (PDGF), endothelin, (ET-1) and TGF-β_2. Slowing epithelial repair with an EGFR selective inhibitor augments release of TGF-β_2 which plays a key role in promoting transformation of fibroblasts into myofibroblasts. In vivo, EGFR over expression in asthmatic bronchial epithelium is positively correlated with the thickness of the *Lamina reticularis* linking epithelial injury to underlying remodelling[163]. In mild-moderate asthma, inhaled corticosteroids reduce airway inflammation and levels of IGF-1, but provide minimal improvement in BHR and have no effect on TGF-β levels. As corticosteroid treatment reduced inflammation, persistently high TGF-β in BAL fluid most likely derive from the injured and repairing epithelium and associated matrix turnover rather than from eosinophils[40]. Since both epithelial EGFR expression and TGF-β production are refractory to corticosteroids, it is believed that the combined effects of these signalling pathways on the epithelium and underlying mesenchymal cells promote remodelling and explain the incomplete resolution of

lung function with inhaled corticosteroids observed in chronic asthma (Figure 1.4).

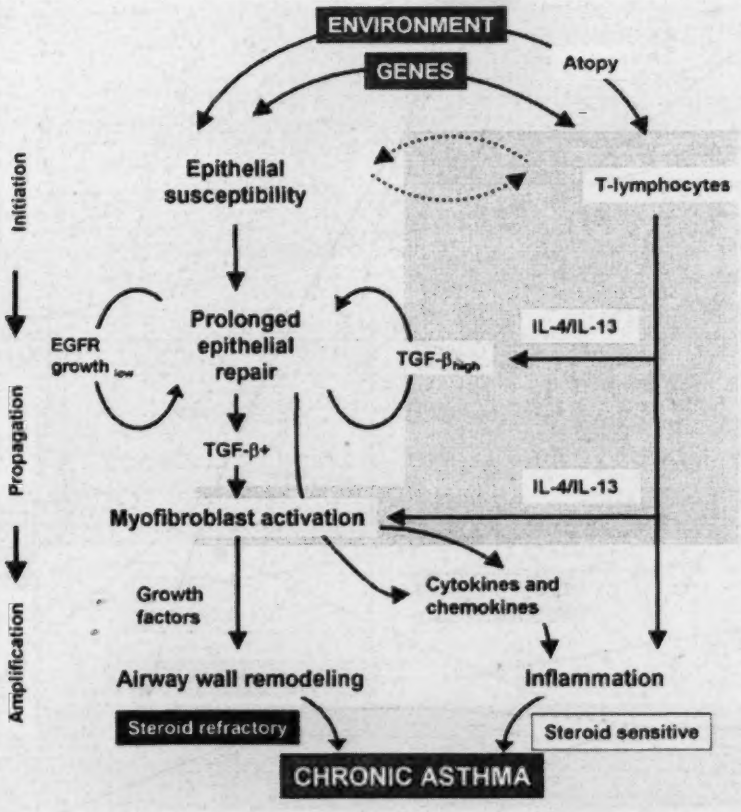


Figure 1.4: The interaction between TH_2 inflammation and the EMTU in asthma pathogenesis. A parallel model for asthma pathogenesis in which an inherited or acquired epithelial susceptibility to environmental agents leads to induction of stress-injury and repair responses is shown. Growth arrest and prolonged repair enhances cell-cell communication within the EMTU, leading to myofibroblast activation and propagation of remodeling responses into the submucosa. At each level, TH_2 cytokines are able to interact with the EMTU to enhance or amplify these responses.

Airway inflammation and remodeling in asthma - cause and effect? Immunologist 2001;8:131-5. (Hogrefe & Huber Publishers, Seattle, Wash)

Although the "EMTU reactivation" paradigm was able to explain many complicated features of asthma. There still remain many unanswered caveats in the paradigm to be addressed in future. One of the most interesting and least known areas is the MMP production and regulation of mediators that control ECM production in asthma. For instance the question remains as to why in the presence of increased level of TGF-β_1 a known factor to strongly inhibit the production of MMP-1 in the lung[165], MMP-1 level are highly increased in asthmatic airways[124]. It is therefore imperative to look for other factors that could play a role in epithelial-mesenchymal communication and could help enhance our understanding of intricate asthma mechanism.

1.8 The 14-3-3 protein family

1.8.1 Introduction

The 14-3-3 protein family was originally identified in 1967 by Moore and Perez during a systematic classification of brain proteins[166]. The name 14-3-3 is derived from the combination of their fraction number after diethylaminoethyl (DEAE)-cellulose chromatogrophy and their migration position after subsequent starch- gel electrophoresis. This class of proteins have been found in all eukaryotic organisms studied so far. There are seven known mammalian 14-3-3 isotypes ($\beta, \gamma, \varepsilon, \sigma, \zeta, \tau$ and η)[167], the σ isotype is also known as stratifin. Two isotypes initially designated as α and δ are the phosphorylated forms of β and ζ[168]. 14-3-3 proteins exist mainly as homo-and heterodimers with monomeric

molecular mass of approximately 30 kDa and an acidic isoelectric point of 4-5 [169]. 14-3-3 is abundant in the brain, comprising approximately 1% of its total soluble protein[170]. It is now clear that 14-3-3 is also present in almost all tissues, including testes, liver, and heart[171]. Within a eukaryotic cell, 14-3-3 is largely found in the cytoplasmic compartment. However, 14-3-3 proteins can also be detected at the plasma membrane and in intracellular organelles such as the nucleus and the Golgi apparatus[171-175].

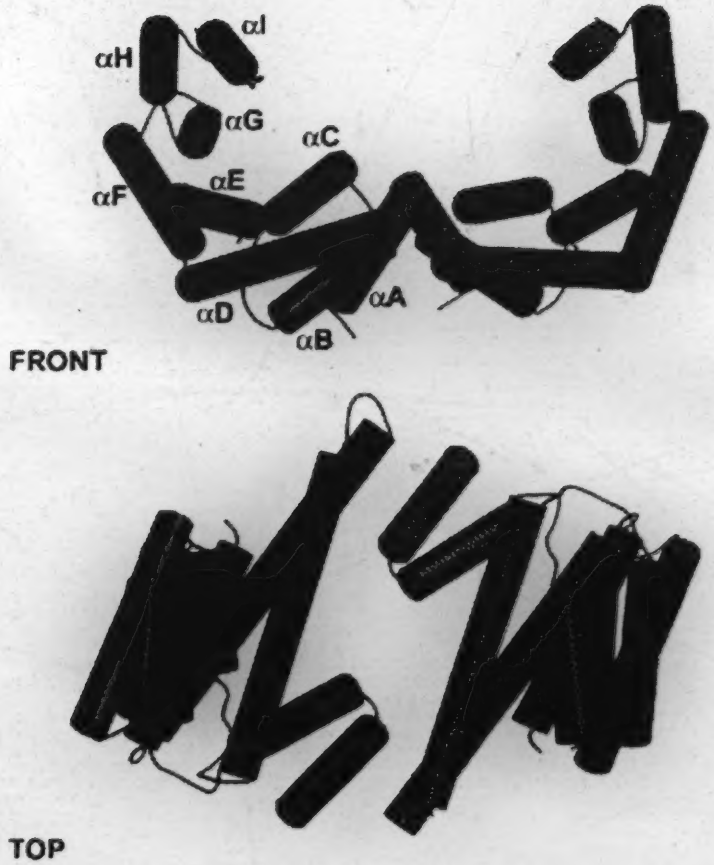
The history of 14-3-3 proteins has witnessed repeated discovery of the same molecule by a number of laboratories. Characterization of a protein cofactor that activates tryptophan and tyrosine hydroxylases uncovered the first function of 14-3-3 proteins[176] and led to the cloning of the first 14-3-3 gene[177]. Thereafter, availability of a 14-3-3 sequence set the stage for a flood of rediscoveries by investigators interested in a wide range of biological questions.

14-3-3s have emerged as a group of multifunctional proteins that bind to and modulate a wide array of cellular proteins involved in processes such as metabolism, signal transduction, cell-cycle control, apoptosis, cytoskeletal structure, protein trafficking, transcription, stress response, and malignant transformation.

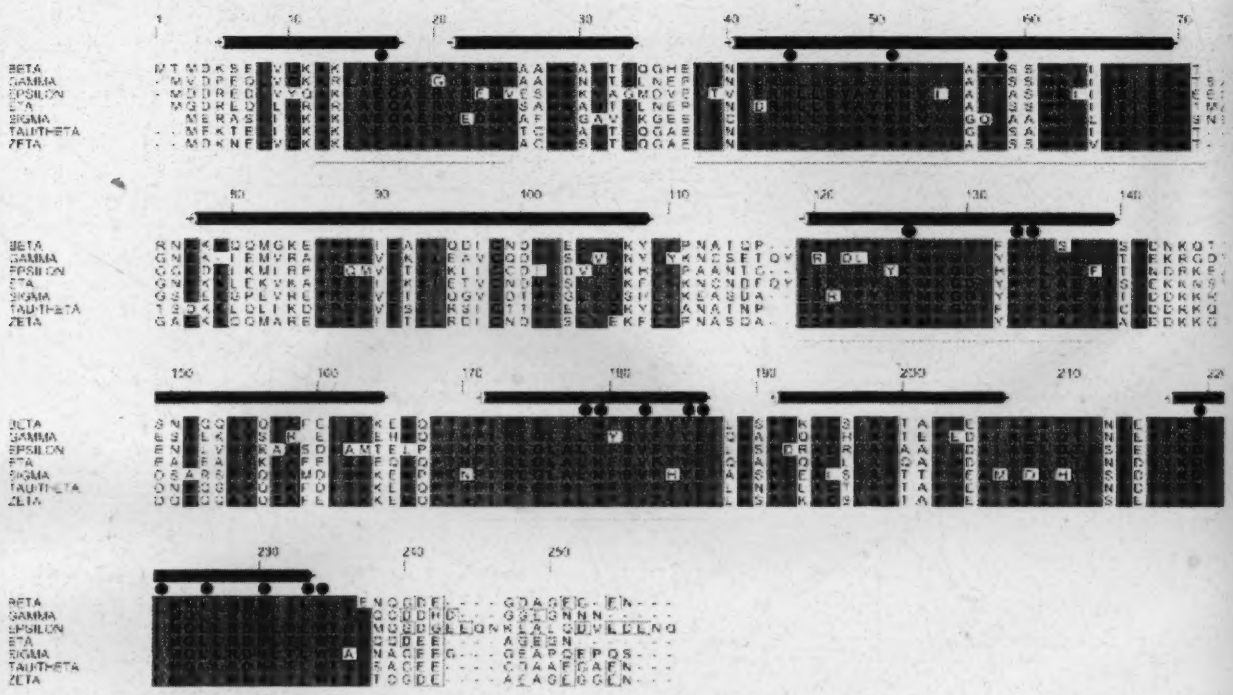
1.8.2 14-3-3 Structure

Identification of the various isotypes crystal structure of 14-3-3, revealed that they are highly helical, dimeric proteins[178,179]. Each monomer is composed of 9 antiparallel α-helices (denoted αA-αI) with the dimer interface formed from

helices αA, αC, and αD (figure 1.5). Despite their genetic diversity, 14-3-3 isotypes are functionally highly homologous proteins, their protein sequence of 14-3-3 proteins consists of five highly conserved blocks that are separated by less conserved regions[180] (Figure 1.6). The conserved regions form the dimer interface as well as line the central ligand binding channel of the dimeric 14-3-3 molecule, thereby are important in peptide binding and dimerization[181]. The dimeric structure of 14-3-3 creates a large negatively charged channel[169]. The invariable regions of all the isotypes are mainly found lining the interior of this channel, while the variable residues are located on the surface of the protein. The residues involved in dimerization are 5-21 in the A-helix of one subunit and residues 58-89 of the C and D helices of the other[182].



(Figure 1.5): The structure of 14-3-3 in two orthogonal views. Helices αA–αI are shown as cylinders. *Yaffe MB. FEBS letters, Issue 1, 513:53-57, 2002*



(Figure 1.6): Sequence alignment of human 14-3-3 isotypes. Residues conserved in at least six of the seven isotypes are shaded gray. The structure of 14-3-3 is indicated by helices above the alignment. Five conserved sequence blocks within the 14-3-3 family, as defined by Wang and Shakes, are indicated by a thin line below the alignment. Residues within the binding cleft that interact with peptide ligands or with the serotonin *N*-acetyl transferase molecule are indicated by filled circles. Acidic residues within the divergent C-termini are boxed. *Yaffe MB. FEBS letter 513:53-57, 2002*

The dimeric structure of 14-3-3 allows the protein to bind two ligands simultaneously[183]. Previous studies have suggested that 14-3-3 proteins may serve as a novel type of "adapter proteins", mediating interactions from different proteins on each subunit of the dimer. For example, the ability of diverse members of this family to form heterodimers between ε and ζ and between τ and ζ isotypes[184] may allow the interaction between signalling proteins that do not associate directly with each other.

1.8.3 14-3-3 Function

The initial introduction of 14-3-3 into some biological systems was based on functional studies aimed at identifying regulatory proteins. For instance, the isolation of inhibitors of protein kinase C (PKC)[185], the identification of stimulators of calcium-dependent exocytosis[186], and the cloning of the eukaryotic activator of the *Pseudomonas aeruginosa* ExoS, ADP-ribosyltransferase[187], each resulted in the rediscovery of the 14-3-3 proteins. Similarly, 14-3-3 has been shown to be an activator of the 43-kDa inositol polyphosphate 5-phosphatase (5-phosphatase) where 14-3-3 functions as an allosteric cofactor to affect the catalytic activity of its ligand[188].

Another major avenue of rediscovery of 14-3-3 accompanied technological advances in detecting protein-protein interactions. In recent years, many signal transduction pathways have been unveiled that control cell proliferation, differentiation, and apoptosis, but understanding the intricate mechanisms that

regulate these pathways remains a daunting challenge[189]. Many investigators search for clues by identifying proteins that interact with key signaling components. 14-3-3 proteins are easy prey for a variety of bait proteins in a large array of these screens. Some examples of 14-3-3 associated proteins include receptors such as Insulin-like growth factor I receptor (IGFIR)[190,191], glucocorticoid receptor[192] and parathyroid hormone receptor[193], kinases such as Raf-1[175,194-196] Bcr[197], and phosphatidylinositol 3 kinase[198], phosphatases such as Cdc25[199] and protein-tyrosine phosphatase(PTHP)[200]. Moreover docking molecules for example, insulin receptor substrate I[201] and p130[Cas 202] as well as death regulators such as Bad[196] and A20[203] and oncogene products like polyomavirus middle tumor antigen (MT)[204] and Bcr-Abl[197] are among the known 14-3-3 associated proteins.

Taken together the frequent isolation of 14-3-3 from many biochemical and genetic screens for different targets must reflect the physiological importance of 14-3-3 in diverse cellular pathways. Depending on its interaction with specific effectors, 14-3-3 participates in many vital regulatory processes, such as cell cycle control, survival signaling, cell adhesion, and neuronal plasticity. The trend of rediscovery of 14-3-3 proteins shows no sign of diminishing and in fact will likely become more common because of an increased understanding of the regulation of 14-3-3 ligand interactions.

1.8.4 14-3-3 Target binding

The heterogeneity and sheer number of binding partners for 14-3-3 allows the prediction of some properties of the interaction. A natural conclusion that can be drawn is that 14-3-3 ligands share a common binding determinant that mediates their contact with 14-3-3. One such determinant is a specifically phosphorylated residue in 14-3-3 ligands. Several early observations guided the realization that phosphorylation of target proteins is the primary mechanism that controls 14-3-3 binding[205]. Analyses of known 14-3-3-binding sites, together with the use of peptide libraries, have defined two (mode 1 and 2) high-affinity phosphorylation-dependent binding motifs (each containing 6 amino acids) that are recognized by all 14-3-3 isotypes[206]: R(S/Ar)(+)pSXP and RX(Ar)(+)pSXP , where pS is phosphoserine, Ar is an aromatic residue (particularly Tyr or Phe in mode 2) and + is a basic amino acid. Residue X following the phosphoserine is commonly Leu, Glu, Ala or Met. The phosphorylated residue may also be a threonine. It is worth noting that there are however, other phosphorylation-dependent sites that diverge significantly from these motifs[182], and it should be noted that some 14-3-3 interactions are independent of phosphorylation. For example, binding of 14-3-3 to exoenzyme S of pseudomonas aeruginosa[207] and to R18 a group of inhibitor peptides[208] does not require a phosphorylated residue. Regardless of whether the interactions are dependent on phosphorylation or not, all targets appear to interact with the same binding domain on 14-3-3[208].

1.8.5 14-3-3 is a regulator of intracellular protein localization

One important means by which 14-3-3 regulates cellular processes is by modulating protein localization[209]. In most cases, 14-3-3 binding sequesters the target protein in a particular subcellular compartment, and the release of 14-3-3 then allows the target to relocate. This relocation is often due to the exposure of an intrinsic subcellular targeting sequence that was masked by the 14-3-3 dimer. This mechanism of regulation contributes to the nuclear retention of proteins such as human telomerase reverse transcriptase (TERT)[210], Txl-2[211] and Chk1[212], and plays a crucial role in the cytoplasm-mitochondrion shuttling of BAD and BAX[196,213] and in the cytoplasm-nucleus shuttling of proteins including Cdc25[214,215], histone deacetylase[216], and the forkhead transcription factors[217,218]. More recently, it has become apparent that this regulatory mechanism also applies to proteins that shuttle from the cytoplasm to the plasma membrane, particularly proteins that are involved in Ras and heterotrimeric G-protein signaling. Heterotrimeric G-proteins and members of the Ras family are membrane-bound; however, many of their effectors and regulators are localized to the cytoplasm and, in order to exert their effects, these molecules must translocate to the cell surface. Various components of these pathways interact with 14-3-3, and more-detailed analyses of these interactions have revealed that, when 14-3-3 binding is disrupted, many of these molecules exhibit increased levels of association with the plasma membrane. This enhanced membrane localization has been observed for the Ras pathway components Raf-1[219], Rin1[220], KSR1[221] and p90 Rsk[222], and the G-protein regulators RGS3[223] and

RGS7[224]. Apart from the above mentioned mechanisms 14-3-3 has also been implicated in the endoplasmic reticulum (ER)-to-plasma-membrane trafficking of certain multimeric complexes, including the KCNK and K_{ATP} potassium channels[225,226]. During forward transport, a trafficking checkpoint prevents the release of unassembled or incomplete forms of these complexes from the ER.

1.8.6 Extracellular function of 14-3-3

Although 14-3-3 proteins have been identified as part of the brain-specific proteins in cerebrospinal fluid(CSF), since 1965 by Moore *et al* [227] and their presence have been associated with many neurological disorders such as Creutzfeldt-Jakob disease[228], Multiple Sclerosis[229] and Alzheimer's disease[230], their presence in the extracellular milieu was attributed to neuronal cell death. Thus far, all the biological activities of 14-3-3 have been identified as intracellular interactions and functions. Recently, Ghahary *et al* (2004) have demonstrated a novel extracellular function for the σ-isotype of 14-3-3[231]. 14-3-3σ isolated from keratinocytes conditioned medium was shown to stimulate MMP-1 production in dermal fibroblasts. Considering that this keratinocyte-releasable factor has a potent collagenase stimulatory effect on fibroblasts, which favors the resolution of accumulated type I and type III collagen found in fibrotic tissue, these investigators referred to this protein as a keratinocyte-derived anti-fibrogenic factor (KDAF). Further, stratifin gene was cloned and its corresponding protein was expressed in *E.coli*. The MMP-1 stimulatory effect of the recombinant 14-3-3σ was then validated in a fibroblast culture system.

Communication of keratinocytes and fibroblasts via a known intracellular mediator such as 14-3-3 marks an important step in establishing yet another role for this multifunctional family of proteins, which also involves the epithelial-mesenchymal trophic unit.

1.9 Thesis Aim and Experimental Rationale

One of the main questions in asthma research is why and how an initial disorder of reversible airway obstruction could progress to a subsequent condition with irreversible airway obstruction. Recent studies show that aberrant signaling between the epithelial and mesenchymal layer could ultimately result in such irreversible changes collectively known as airway remodeling.

As discussed earlier our laboratory has recently discovered 14-3-3s released from keratinocytes to be an important factor that upregulates MMP-1 production in dermal fibroblasts. Subsequently, 14-3-3α/β was also identified in the keratinocyte conditioned medium (KCM), with MMP-1 stimulatory effect in fibroblasts.

Increased level of MMP-1 in asthmatic airways has long been documented and has been associated with the impaired cycles of degradation and repair in the airways as well as some specific remodeling features such as smooth muscle hypertrophy. The discovery of KDAF as an important mediator in epithelial-mesenchymal communication brought about many interesting questions about the potential contribution of this factor in other organs of the body.

Three related questions remain; first, whether or not keratinocytes are the only epithelial cells capable of releasing 14-3-3 proteins? If not, is 14-3-3 capable of modulating MMP-1 production in different mesenchymal cells other than dermal fibroblasts? As such, are other isotypes of 14-3-3 important in MMP-1 regulation in different cell types?

Based on above, in this study, I hypothesize that lung epithelial cells are able to release extracellular 14-3-3β protein which can enhance the MMP-1 expression in lung fibroblasts.

Therefore, the specific aims of the study were to:

I. Identify the presence of 14-3-3 proteins in lung epithelial-cell conditioned medium (LCM).

II. Study and compare the effect of LCM to that of recombinant 14-3-3β and σ on lung fibroblast in an epithelial-fibroblast co-culture system.

III. Compare the effect of 14-3-3β on MMP-1 expression to that of TGF-β, a known factor that reduces MMP-1 expression in lung fibroblasts.

IV. Study the effect of TH2 cytokines on 14-3-3 expression in lung epithelial cells.

1.10 Reference List

1. Salter HH. The pathology of asthma-- its absolute nature. *Brewis RAL* 106-142 (2004).

2. Ellis AG. The pathologic anatomy of bronchial asthma. *Am.J.Med.Sci* 407-410 (2004).

3. Kim, J., Woods, A., Beckerdunn, E., & Bottomly, K. Distinct Functional Phenotypes of Cloned Ia-Restricted Helper T-Cells. *Journal of Experimental Medicine* **162**, 188-201 (1985).

4. Mosmann, T. R., Cherwinski, H., Bond, M. W., Giedlin, M. A., & Coffman, R. L. Two types of murine helper T cell clone. I. Definition according to profiles of lymphokine activities and secreted proteins. *J.Immunol.* **136**, 2348-2357 (1986).

5. *Rep.* No. NIH publication no. 97-4051, 2004).

6. Djukanovic, R. Bronchoscopy as a research tool for the study of asthma pathogenesis and effects of antiasthma drugs. *J.Allergy Clin.Immunol.* **98**, S41-S45 (1996).

7. Djukanovic, R., Dahl, R., Jarjour, N., & Aalbers, R. Safety of biopsies and bronchoalveolar lavage. *Eur.Respir.J.Suppl* **26**, 39S-41S (1998).

8. Busse, W. W. & Lemanske, R. F., Jr. Asthma. *N.Engl.J Med* **344**, 350-362 (2001).

9. Bentley, A. M. *et al.* Identification of T lymphocytes, macrophages, and activated eosinophils in the bronchial mucosa in intrinsic asthma. Relationship to symptoms and bronchial responsiveness. *Am.Rev.Respir.Dis.* **146**, 500-506 (1992).

10. Antczak, A., Montuschi, P., Kharitonov, S., Gorski, P., & Barnes, P. J. Increased exhaled cysteinyl-leukotrienes and 8-isoprostane in aspirin-induced asthma. *Am J Respir Crit Care Med* **166**, 301-306 (2002).

11. *Rep.* No. NHLBI publication no. 02-3659:93-172, 2004).

12. Van Ganse, E. *et al.* Persistent asthma: disease control, resource utilisation and direct costs. *Eur.Respir.J.* **20**, 260-267 (2002).

13. Holgate, S. T. Asthma: more than an inflammatory disease. *Curr.Opin.Allergy Clin.Immunol.* **2**, 27-29 (2002).

14. Bryan, S. A. *et al.* Effects of recombinant human interleukin-12 on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* **356**, 2149-2153 (2000).

15. Leckie, M. J. *et al.* Effects of an interleukin-5 blocking monoclonal antibody on eosinophils, airway hyper-responsiveness, and the late asthmatic response. *Lancet* **356**, 2144-2148 (2000).

16. Flood-Page M *et al.* Reduction of tissue eosinophils in mild atopic asthmatics by an ant-IL-5 monoclonal antibody (mepolizumab) is associated with inhibition of tenascin deposition within the bronchial epithelial basement membrane [abstract]. *Am J Respir Crit Care Med* 165: B42 (2004).

17. Skadhauge, L. R., Christensen, K., Kyvik, K. O., & Sigsgaard, T. Genetic and environmental influence on asthma: a population-based study of 11,688 Danish twin pairs. *Eur.Respir J* **13**, 8-14 (1999).

18. Holgate, S. T. Genetic and environmental interaction in allergy and asthma. *J Allergy Clin.Immunol.* **104**, 1139-1146 (1999).

19. Rahman, I. & MacNee, W. Oxidative stress and regulation of glutathione in lung inflammation. *Eur.Respir J* **16**, 534-554 (2000).

20. Migliaretti, G. & Cavallo, F. Urban air pollution and asthma in children. *Pediatr.Pulmonol.* **38**, 198-203 (2004).

21. Soutar, A., Seaton, A., & Brown, K. Bronchial reactivity and dietary antioxidants. *Thorax* **52**, 166-170 (1997).

22. Message, S. D. & Johnston, S. L. Viruses in asthma. *Br.Med Bull.* **61**, 29-43 (2002).

23. Cohn, L., Elias, J. A., & Chupp, G. L. Asthma: mechanisms of disease persistence and progression. *Annu.Rev.Immunol.* **22**, 789-815 (2004).

24. Davies, D. E., Wicks, J., Powell, R. M., Puddicombe, S. M., & Holgate, S. T. Airway remodeling in asthma: new insights. *J Allergy Clin.Immunol.* **111**, 215-225 (2003).

25. Dunnill, M. S. The pathology of asthma. *Ciba Found.Study Group* **38**, 35-46 (1971).

26. Busse, W. W., Banks-Schlegel, S., & Wenzel, S. E. Pathophysiology of severe asthma. *J Allergy Clin.Immunol.* **106**, 1033-1042 (2000).

27. Jenkins, H. A. *et al.* Histopathology of severe childhood asthma: a case series. *Chest* **124**, 32-41 (2003).

28. Lazaar, A. L. & Panettieri, R. A., Jr. Is airway remodeling clinically relevant in asthma? *Am J Med* **115**, 652-659 (2003).

29. Xu, J. *et al.* Matrix metalloproteinase-2 from bronchial epithelial cells induces the proliferation of subepithelial fibroblasts. *Clin.Exp.Allergy* **32**, 881-888 (2002).

30. Hamilton, L. M. *et al.* The bronchial epithelium in asthma--much more than a passive barrier. *Monaldi Arch.Chest Dis.* **56**, 48-54 (2001).

31. Jeffery, P. K., Wardlaw, A. J., Nelson, F. C., Collins, J. V., & Kay, A. B. Bronchial biopsies in asthma. An ultrastructural, quantitative study and correlation with hyperreactivity. *Am Rev.Respir Dis.* **140**, 1745-1753 (1989).

32. Beasley, R., Roche, W. R., Roberts, J. A., & Holgate, S. T. Cellular events in the bronchi in mild asthma and after bronchial provocation. *Am Rev.Respir Dis.* **139**, 806-817 (1989).

33. Roche, W. R., Beasley, R., Williams, J. H., & Holgate, S. T. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* **1**, 520-524 (1989).

34. Puddicombe, S. M. *et al.* Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *Faseb Journal* **14**, 1362-1374 (2000).

35. Puddicombe, S. M. *et al.* Increased expression of p21(waf) cyclin-dependent kinase inhibitor in asthmatic bronchial epithelium. *Am J Respir Cell Mol.Biol.* **28**, 61-68 (2003).

36. Demoly, P. *et al.* Cell proliferation in the bronchial mucosa of asthmatics and chronic bronchitics. *Am J Respir Crit Care Med* **150**, 214-217 (1994).

37. Redington, A. E. *et al.* Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* **156**, 642-647 (1997).

38. Redington, A. E., Roche, W. R., Holgate, S. T., & Howarth, P. H. Co-localization of immunoreactive transforming growth factor-beta 1 and decorin in bronchial biopsies from asthmatic and normal subjects. *J Pathol* **186**, 410-415 (1998).

39. Hamilton, L. M. *et al.* The role of the epidermal growth factor receptor in sustaining neutrophil inflammation in severe asthma. *Clinical and Experimental Allergy* **33**, 233-240 (2003).

40. Holgate, S. T. *et al.* Invited lecture: activation of the epithelial mesenchymal trophic unit in the pathogenesis of asthma. *Int.Arch.Allergy Immunol.* **124**, 253-258 (2001).

41. Laitinen, A. *et al.* Tenascin is increased in airway basement membrane of asthmatics and decreased by an inhaled steroid. *Am J Respir Crit Care Med* **156**, 951-958 (1997).

42. Johnson, P. R. Role of human airway smooth muscle in altered extracellular matrix production in asthma. *Clin.Exp.Pharmacol.Physiol* **28**, 233-236 (2001).

43. Roche, W. R. Fibroblasts and asthma. *Clin.Exp.Allergy* **21**, 545-548 (1991).

44. Chakir, J. *et al.* Lower airways remodeling in nonasthmatic subjects with allergic rhinitis. *Lab Invest* **75**, 735-744 (1996).

45. Chu, H. W. *et al.* Collagen deposition in large airways may not differentiate severe asthma from milder forms of the disease. *Am J Respir Crit Care Med* **158**, 1936-1944 (1998).

46. Laitinen, L. A. *et al.* Bronchial biopsy findings in intermittent or "early" asthma. *J Allergy Clin.Immunol.* **98**, S3-S6 (1996).

47. Boulet, L. P. *et al.* Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med* **162**, 1308-1313 (2000).

48. Payne, D. N. *et al.* Early thickening of the reticular basement membrane in children with difficult asthma. *Am J Respir Crit Care Med* **167**, 78-82 (2003).

49. Chetta, A. *et al.* Airways remodeling is a distinctive feature of asthma and is related to severity of disease. *Chest* **111**, 852-857 (1997).

50. Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J., & Isogai, S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin.Immunol.* **102**, 783-788 (1998).

51. Minshall, E. M. *et al.* Eosinophil-associated TGF-beta1 mRNA expression and airways fibrosis in bronchial asthma. *Am J Respir Cell Mol.Biol.* **17**, 326-333 (1997).

52. Wilson, J. W. & Li, X. The measurement of reticular basement membrane and submucosal collagen in the asthmatic airway. *Clin.Exp.Allergy* **27**, 363-371 (1997).

53. Roberts, C. R. Is asthma a fibrotic disease? *Chest* **107**, 111S-117S (1995).

54. Frey, U., Makkonen, K., Wellman, T., Beardsmore, C., & Silverman, M. Alterations in airway wall properties in infants with a history of wheezing disorders. *Am J Respir Crit Care Med* **161**, 1825-1829 (2000).

55. Johns, D. P., Wilson, J., Harding, R., & Walters, E. H. Airway distensibility in healthy and asthmatic subjects: effect of lung volume history. *J Appl.Physiol* **88**, 1413-1420 (2000).

56. Ward, C. *et al.* Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma. *Am J Respir Crit Care Med* **164**, 1718-1721 (2001).

57. Altraja, A. *et al.* Expression of laminins in the airways in various types of asthmatic patients: a morphometric study. *Am J Respir Cell Mol.Biol.* **15**, 482-488 (1996).

58. Huang, J., Olivenstein, R., Taha, R., Hamid, Q., & Ludwig, M. Enhanced proteoglycan deposition in the airway wall of atopic asthmatics. *Am J Respir Crit Care Med* **160**, 725-729 (1999).

59. Westergren-Thorsson, G., Chakir, J., Lafreniere-Allard, M. J., Boulet, L. P., & Tremblay, G. M. Correlation between airway responsiveness and proteoglycan production by bronchial fibroblasts from normal and asthmatic subjects. *Int.J Biochem.Cell Biol.* **34**, 1256-1267 (2002).

60. Bousquet, J. *et al.* Bronchial elastic fibers in normal subjects and asthmatic patients. *Am J Respir Crit Care Med* **153**, 1648-1654 (1996).

61. Gelb, A. F., Licuanan, J., Shinar, C. M., & Zamel, N. Unsuspected loss of lung elastic recoil in chronic persistent asthma. *Chest* **121**, 715-721 (2002).

62. Wenzel, S. E. *et al.* Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct

physiologic and clinical characteristics. *Am J Respir Crit Care Med* **160**, 1001-1008 (1999).

63. Ordonez, C. L. *et al.* Mild and moderate asthma is associated with airway goblet cell hyperplasia and abnormalities in mucin gene expression. *Am J Respir Crit Care Med* **163**, 517-523 (2001).

64. Groneberg, D. A. *et al.* Expression of respiratory mucins in fatal status asthmaticus and mild asthma. *Histopathology* **40**, 367-373 (2002).

65. Huber, H. L. & Koessler, K. K. The pathology of bronchial asthma. *Arch Intern Med* **30**, 689-760 (2004).

66. Heard, B. E. & Hossain, S. Hyperplasia of bronchial muscle in asthma. *J Pathol* **110**, 319-331 (2004).

67. Amrani, Y., Krymskaya, V., Maki, C., & Panettieri, R. A., Jr. Mechanisms underlying TNF-alpha effects on agonist-mediated calcium homeostasis in human airway smooth muscle cells. *Am J Physiol* **273**, L1020-L1028 (1997).

68. Halayko, A. J. & Stephens, N. L. Potential role for phenotypic modulation of bronchial smooth muscle cells in chronic asthma. *Can.J Physiol Pharmacol.* **72**, 1448-1457 (1994).

69. Halayko, A. J. & Solway, J. Molecular mechanisms of phenotypic plasticity in smooth muscle cells. *J Appl.Physiol* **90**, 358-368 (2001).

70. Lee, J. H. *et al.* Interleukin-13 induces dramatically different transcriptional programs in three human airway cell types. *Am J Respir Cell Mol.Biol.* **25**, 474-485 (2001).

71. Chung, K. F. Airway smooth muscle cells: contributing to and regulating airway mucosal inflammation? *Eur.Respir J* **15**, 961-968 (2000).

72. Ma, X. *et al.* Changes in biophysical and biochemical properties of single bronchial smooth muscle cells from asthmatic subjects. *Am J Physiol Lung Cell Mol.Physiol* **283**, L1181-L1189 (2002).

73. Halayko, A. J., Rector, E., & Stephens, N. L. Airway smooth muscle cell proliferation: characterization of subpopulations by sensitivity to heparin inhibition. *Am J Physiol* **274**, L17-L25 (1998).

74. Halayko, A. J. *et al.* Divergent differentiation paths in airway smooth muscle culture: induction of functionally contractile myocytes. *Am J Physiol* **276**, L197-L206 (1999).

75. Hirst, S. J., Walker, T. R., & Chilvers, E. R. Phenotypic diversity and molecular mechanisms of airway smooth muscle proliferation in asthma. *Eur.Respir J* **16**, 159-177 (2000).

76. Halayko, A. J. & Amrani, Y. Mechanisms of inflammation-mediated airway smooth muscle plasticity and airways remodeling in asthma. *Respir Physiol Neurobiol.* **137**, 209-222 (2003).

77. Salvato, G. Quantitative and morphological analysis of the vascular bed in bronchial biopsy specimens from asthmatic and non-asthmatic subjects. *Thorax* **56**, 902-906 (2001).

78. Li, X. & Wilson, J. W. Increased vascularity of the bronchial mucosa in mild asthma. *Am J Respir Crit Care Med* **156**, 229-233 (1997).

79. Carroll, N. G., Cooke, C., & James, A. L. Bronchial blood vessel dimensions in asthma. *Am J Respir Crit Care Med* **155**, 689-695 (1997).

80. Chu, H. W., Kraft, M., Rex, M. D., & Martin, R. J. Evaluation of blood vessels and edema in the airways of asthma patients: regulation with clarithromycin treatment. *Chest* **120**, 416-422 (2001).

81. Kumar, S. D., Emery, M. J., Atkins, N. D., Danta, I., & Wanner, A. Airway mucosal blood flow in bronchial asthma. *Am J Respir Crit Care Med* **158**, 153-156 (1998).

82. McDonald, D. M. Angiogenesis and remodeling of airway vasculature in chronic inflammation. *Am J Respir Crit Care Med* **164**, S39-S45 (2001).

83. Vrugt, B. *et al.* Bronchial angiogenesis in severe glucocorticoid-dependent asthma. *Eur.Respir J* **15**, 1014-1021 (2000).

84. Tanaka, H. *et al.* Increased airway vascularity in newly diagnosed asthma using a high-magnification bronchovideoscope. *Am J Respir Crit Care Med* **168**, 1495-1499 (2003).

85. Elias, J. A. *et al.* New insights into the pathogenesis of asthma. *J Clin.Invest* **111**, 291-297 (2003).

86. Ying, S., Durham, S. R., Corrigan, C. J., Hamid, Q., & Kay, A. B. Phenotype of cells expressing mRNA for TH2-type (interleukin 4 and interleukin 5) and TH1-type (interleukin 2 and interferon gamma) cytokines in bronchoalveolar lavage and bronchial biopsies from atopic asthmatic and normal control subjects. *Am J Respir Cell Mol.Biol.* **12**, 477-487 (1995).

87. Fort, M. M. *et al.* IL-25 induces IL-4, IL-5, and IL-13 and Th2-associated pathologies in vivo. *Immunity.* **15**, 985-995 (2001).

88. Hoshino, T., Winkler-Pickett, R. T., Mason, A. T., Ortaldo, J. R., & Young, H. A. IL-13 production by NK cells: IL-13-producing NK and T cells are present in vivo in the absence of IFN-gamma. *J Immunol.* **162**, 51-59 (1999).

89. Yoshimoto, T., Bendelac, A., Watson, C., Hu-Li, J., & Paul, W. E. Role of NK1.1+ T cells in a TH2 response and in immunoglobulin E production. *Science* **270**, 1845-1847 (1995).

90. Ferrick, D. A. *et al.* Differential production of interferon-gamma and interleukin-4 in response to Th1- and Th2-stimulating pathogens by gamma delta T cells in vivo. *Nature* **373**, 255-257 (1995).

91. Rothenberg, M. E. Eosinophilia. *N.Engl.J Med* **338**, 1592-1600 (1998).

92. Ikeda, K. *et al.* Mast cells produce interleukin-25 upon Fc epsilon RI-mediated activation. *Blood* **101**, 3594-3596 (2003).

93. Mould, A. W., Matthaei, K. I., Young, I. G., & Foster, P. S. Relationship between interleukin-5 and eotaxin in regulating blood and tissue eosinophilia in mice. *J Clin.Invest* **99**, 1064-1071 (1997).

94. Mattes, J. *et al.* Intrinsic defect in T cell production of interleukin (IL)-13 in the absence of both IL-5 and eotaxin precludes the development of eosinophilia and airways hyperreactivity in experimental asthma. *J Exp.Med* **195**, 1433-1444 (2002).

95. Nakajima, H. *et al.* CD4+ T-lymphocytes and interleukin-5 mediate antigen-induced eosinophil infiltration into the mouse trachea. *Am Rev.Respir Dis.* **146**, 374-377 (1992).

96. Mattes, J. *et al.* IL-13 induces airways hyperreactivity independently of the IL-4R alpha chain in the allergic lung. *J Immunol.* **167**, 1683-1692 (2001).

97. Mathew, A. *et al.* Signal transducer and activator of transcription 6 controls chemokine production and T helper cell type 2 cell trafficking in allergic pulmonary inflammation. *J Exp.Med* **193**, 1087-1096 (2001).

98. Morokata, T., Ishikawa, J., Ida, K., & Yamada, T. C57BL/6 mice are more susceptible to antigen-induced pulmonary eosinophilia than BALB/c mice, irrespective of systemic T helper 1/T helper 2 responses. *Immunology* **98**, 345-351 (1999).

99. Phipps, S. *et al.* The relationship between allergen-induced tissue eosinophilia and markers of repair and remodeling in human atopic skin. *Journal of Immunology* **169**, 4604-4612 (2002).

100. Bousquet, J. *et al.* Eosinophilic inflammation in asthma. *N.Engl.J Med* **323**, 1033-1039 (1990).

101. Walker, C., Kaegi, M. K., Braun, P., & Blaser, K. Activated T cells and eosinophilia in bronchoalveolar lavages from subjects with asthma correlated with disease severity. *J Allergy Clin.Immunol.* **88**, 935-942 (1991).

102. Wardlaw, A. J. *et al.* New insights into the relationship between airway inflammation and asthma. *Clinical Science* **103**, 201-211 (2002).

103. Green, R. H. *et al.* Asthma exacerbations and sputum eosinophil counts: a randomised controlled trial. *Lancet* **360**, 1715-1721 (2002).

104. Driver, A. G., Kukoly, C. A., Ali, S., & Mustafa, S. J. Adenosine in bronchoalveolar lavage fluid in asthma. *Am Rev.Respir Dis.* **148**, 91-97 (1993).

105. Mann, J. S., Holgate, S. T., Renwick, A. G., & Cushley, M. J. Airway effects of purine nucleosides and nucleotides and release with bronchial provocation in asthma. *J Appl.Physiol* **61**, 1667-1676 (1986).

106. Blackburn, M. R. *et al.* Adenosine mediates IL-13-induced inflammation and remodeling in the lung and interacts in an IL-13-adenosine amplification pathway. *J Clin.Invest* **112**, 332-344 (2003).

107. Livingston, M., Heaney, L. G., & Ennis, M. Adenosine, inflammation and asthma--a review. *Inflamm.Res.* **53**, 171-178 (2004).

108. Sousa, A. R. *et al.* Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am J Respir Cell Mol.Biol.* **10**, 142-147 (1994).

109. Zhu, Z. *et al.* IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol.* **168**, 2953-2962 (2002).

110. Zhong, H. *et al.* A(2B) adenosine receptors increase cytokine release by bronchial smooth muscle cells. *Am J Respir Cell Mol.Biol.* **30**, 118-125 (2004).

111. Carr, M. W., Roth, S. J., Luther, E., Rose, S. S., & Springer, T. A. Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc.Natl.Acad.Sci U.S.A* **91**, 3652-3656 (1994).

112. Karpus, W. J. *et al.* Differential CC chemokine-induced enhancement of T helper cell cytokine production. *J Immunol.* **158**, 4129-4136 (1997).

113. Gu, L. *et al.* Control of TH2 polarization by the chemokine monocyte chemoattractant protein-1. *Nature* **404**, 407-411 (2000).

114. Lu, B. *et al.* Abnormalities in monocyte recruitment and cytokine expression in monocyte chemoattractant protein 1-deficient mice. *J Exp.Med* **187**, 601-608 (1998).

115. Traynor, T. R., Kuziel, W. A., Toews, G. B., & Huffnagle, G. B. CCR2 expression determines T1 versus T2 polarization during pulmonary Cryptococcus neoformans infection. *J Immunol.* **164**, 2021-2027 (2000).

116. Conti, P. *et al.* Monocyte chemotactic protein-1 provokes mast cell aggregation and [3H]5HT release. *Immunology* **86**, 434-440 (1995).

117. Hogaboam, C. M. *et al.* Differential monocyte chemoattractant protein-1 and chemokine receptor 2 expression by murine lung fibroblasts derived from Th1- and Th2-type pulmonary granuloma models. *J Immunol.* **163**, 2193-2201 (1999).

118. Smith, R. E., Strieter, R. M., Phan, S. H., & Kunkel, S. L. C-C chemokines: novel mediators of the profibrotic inflammatory response to bleomycin challenge. *Am J Respir Cell Mol.Biol.* **15**, 693-702 (1996).

119. Zhu, Z. *et al.* IL-13-induced chemokine responses in the lung: role of CCR2 in the pathogenesis of IL-13-induced inflammation and remodeling. *J Immunol.* **168**, 2953-2962 (2002).

120. Nagase, H. & Woessner, J. F. Matrix metalloproteinases. *Journal of Biological Chemistry* **274**, 21491-21494 (1999).

121. Primakoff, P. & Myles, D. G. The ADAM gene family - surface proteins with adhesion and protease activity. *Trends in Genetics* **16**, 83-87 (2000).

122. Kaushal, G. P. & Shah, S. V. The new kids on the block: ADAMTSs, potentially multifunctional metalloproteinases of the

ADAM family. *Journal of Clinical Investigation* **105**, 1335-1337 (2000).

123. Madri, J. A. & Graesser, D. Cell migration in the immune system: the evolving inter-related roles of adhesion molecules and proteinases. *Dev.Immunol.* **7**, 103-116 (2000).

124. Kelly, E. A. & Jarjour, N. N. Role of matrix metalloproteinases in asthma. *Curr.Opin.Pulm.Med* **9**, 28-33 (2003).

125. Cataldo, D. D. *et al.* Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases mRNA transcripts in the bronchial secretions of asthmatics. *Lab Invest* **84**, 418-424 (2004).

126. Atkinson, J. J. & Senior, R. M. Matrix metalloproteinase-9 in lung remodeling. *Am J Respir Cell Mol.Biol.* **28**, 12-24 (2003).

127. Kelly, E. A., Busse, W. W., & Jarjour, N. N. Increased matrix metalloproteinase-9 in the airway after allergen challenge. *Am J Respir Crit Care Med* **162**, 1157-1161 (2000).

128. Mautino, G., Capony, F., Bousquet, J., & Vignola, A. M. Balance in asthma between matrix metalloproteinases and their inhibitors. *J Allergy Clin.Immunol.* **104**, 530-533 (1999).

129. Lanone, S. *et al.* Overlapping and enzyme-specific contributions of matrix metalloproteinases-9 and -12 in IL-13-induced inflammation and remodeling. *J Clin.Invest* **110**, 463-474 (2002).

130. Corry, D. B. *et al.* Decreased allergic lung inflammatory cell egression and increased susceptibility to asphyxiation in MMP2-deficiency. *Nat.Immunol.* **3**, 347-353 (2002).

131. McQuibban, G. A. *et al.* Matrix metalloproteinase processing of monocyte chemoattractant proteins generates CC chemokine receptor antagonists with anti-inflammatory properties in vivo. *Blood* **100**, 1160-1167 (2002).

132. Sheppard, D. Killed by leukocytes that don't know when to leave. *Nat.Immunol.* **3**, 337-338 (2002).

133. Emonard H, Calle A, Grimaud JA, & et al. Interactions between fibroblasts and a reconstituted basement membrane matrix. *J Invest Dermatol* 156-163 (2004).

134. Finlay, G. A. *et al.* Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am.J Respir.Crit Care Med.* **156**, 240-247 (1997).

135. Pilcher, B. K., Sudbeck, B. D., Dumin, J. A., Welgus, H. G., & Parks, W. C. Collagenase-1 and collagen in epidermal repair. *Arch.Dermatol Res.* **290 Suppl**, S37-S46 (1998).

136. Fowlkes, J. L., Enghild, J. J., Suzuki, K., & Nagase, H. Matrix Metalloproteinases Degrade Insulin-Like Growth Factor-Binding Protein-3 in Dermal Fibroblast-Cultures. *Journal of Biological Chemistry* **269**, 25742-25746 (1994).

137. Noveral, J. P., Bhala, A., Hintz, R. L., Grunstein, M. M., & Cohen, P. Insulin-like growth factor axis in airway smooth muscle cells. *Am J Physiol* **267**, L761-L765 (1994).

138. Jones, J. I. & Clemmons, D. R. Insulin-like growth factors and their binding proteins: biological actions. *Endocr.Rev.* **16**, 3-34 (1995).

139. Gockerman, A., Prevette, T., Jones, J. I., & Clemmons, D. R. Insulin-like growth factor (IGF)-binding proteins inhibit the smooth muscle cell migration responses to IGF-I and IGF-II. *Endocrinology* **136**, 4168-4173 (1995).

140. Rajah, R. *et al.* Elevated levels of the IGF-binding protein protease MMP-1 in asthmatic airway smooth muscle. *Am J Respir Cell Mol.Biol.* **20**, 199-208 (1999).

141. Fukuda, K. *et al.* Application of efficient and specific gene transfer systems and organ culture techniques for the elucidation of mechanisms of epithelial-mesenchymal interaction in the developing gut. *Dev.Growth Differ.* **42**, 207-211 (2000).

142. MacDonald, M. E. & Hall, B. K. Altered timing of the extracellular-matrix-mediated epithelial-mesenchymal interaction that initiates mandibular skeletogenesis in three inbred strains of mice: development, heterochrony, and evolutionary change in morphology. *J Exp.Zool.* **291**, 258-273 (2001).

143. Wallner, E. I., Yang, Q., Peterson, D. R., Wada, J., & Kanwar, Y. S. Relevance of extracellular matrix, its receptors, and cell adhesion molecules in mammalian nephrogenesis. *Am J Physiol* **275**, F467-F477 (1998).

144. Francis-West, P., Ladher, R., Barlow, A., & Graveson, A. Signalling interactions during facial development. *Mech.Dev.* **75**, 3-28 (1998).

145. Stewart, F. Roles of mesenchymal-epithelial interactions and hepatocyte growth factor-scatter factor (HGF-SF) in placental development. *Rev.Reprod.* **1**, 144-148 (1996).

146. Warburton, D., Zhao, J., Berberich, M. A., & Bernfield, M. Molecular embryology of the lung: then, now, and in the future. *Am J Physiol* **276**, L697-L704 (1999).

147. Warburton, D. *et al.* The molecular basis of lung morphogenesis. *Mechanisms of Development* **92**, 55-81 (2000).

148. Alescio, T. & Piperno, E. C. A Quantitative Assessment of Mesenchymal Contribution to Epithelial Growth Rate in Mouse Embryonic Lung Developing in Vitro. *Journal of Embryology and Experimental Morphology* **17**, 213-& (1967).

149. Muraoka, R. S., Bushdid, P. B., Brantley, D. M., Yull, F. E., & Kerr, L. D. Mesenchymal expression of nuclear factor-kappa B inhibits epithelial growth and branching in the embryonic chick lung. *Developmental Biology* **225**, 322-338 (2000).

150. Warburton, D. *et al.* Growth factor signaling in lung morphogenetic centers: automaticity, stereotypy and symmetry. *Respiratory Research* **4**, (2003).

151. Guarino, N., Solari, V., Shima, H., & Puri, P. Upregulated expression of EGF and TGF-alpha in the proximal respiratory epithelium in the human hypoplastic lung in congenital diaphragmatic hernia. *Pediatr.Surg.Int.* **19**, 755-759 (2004).

152. Zhao, J. S. *et al.* Abrogation of transforming growth factor-beta type II receptor stimulates embryonic mouse lung branching morphogenesis in culture. *Developmental Biology* **180**, 242-257 (1996).

153. Pepicelli, C. V., Lewis, P. M., & McMahon, A. P. Sonic hedgehog regulates branching morphogenesis in the mammalian lung. *Current Biology* **8**, 1083-1086 (1998).

154. Morrisey, E. E. Wnt signaling and pulmonary fibrosis. *American Journal of Pathology* **162**, 1393-1397 (2003).

155. Nicolai, T. Environmental air pollution and lung disease in children. *Monaldi Arch Chest Dis.* **54**, 475-478 (1999).

156. Stein, R. T. *et al.* Respiratory syncytial virus in early life and risk of wheeze and allergy by age 13 years. *Lancet* **354**, 541-545 (1999).

157. Chung, K. F. & Barnes, P. J. Cytokines in asthma. *Thorax* **54**, 825-857 (1999).

158. Montefort, S., Roberts, J. A., Beasley, R., Holgate, S. T., & Roche, W. R. The Site of Disruption of the Bronchial Epithelium in Asthmatic and Nonasthmatic Subjects. *Thorax* **47**, 499-503 (1992).

159. Chen, K., Vita, J. A., Berk, B. C., & Keaney, J. F. c-jun N-terminal kinase activation by hydrogen peroxide in endothelial cells involves Src-dependent epidermal growth factor receptor transactivation. *Journal of Biological Chemistry* **276**, 16045-16050 (2001).

160. Sampath, D., Castro, M., Look, D. C., & Holtzman, M. J. Constitutive activation of an epithelial signal transducer and activator of transcription (STAT) pathway in asthma. *Journal of Clinical Investigation* **103**, 1353-1361 (1999).

161. Mullings, R. E. *et al.* Signal transducer and activator of transcription 6 (STAT-6) expression and function in asthmatic bronchial epithelium. *Journal of Allergy and Clinical Immunology* **108**, 832-838 (2001).

162. Nakao, A. Is TGF-beta1 the key to suppression of human asthma? *Trends Immunol.* **22**, 115-118 (2001).

163. Davies, D. E. & Holgate, S. T. Asthma: the importance of epithelial mesenchymal communication in pathogenesis - Inflammation and the airway epithelium in asthma. *International Journal of Biochemistry & Cell Biology* **34**, 1520-1526 (2002).

164. Zhang, S. L., Smartt, H., Holgate, S. T., & Roche, W. R. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: An in vitro co-culture model of airway remodeling in asthma. *Laboratory Investigation* **79**, 395-405 (1999).

165. Eickelberg, O. *et al.* Extracellular matrix deposition by primary human lung fibroblasts in response to TGF-beta1 and TGF-beta3. *Am J Physiol* **276**, L814-L824 (1999).

166. Moore, B. W. & Perez, V. J. First description of 14-3-3 proteins in mammalian brains. *Physiological and Biochemical Aspect of Nervous Integration* 343-359 (2004).

167. Martin, H. *et al.* Antibodies against the major brain isoforms of 14-3-3 protein. An antibody specific for the N-acetylated amino-terminus of a protein. *FEBS Lett.* **331**, 296-303 (1993).

168. Aitken, A., Howell, S., Jones, D., Madrazo, J., & Patel, Y. 14-3-3 alpha and delta are the phosphorylated forms of raf-activating 14-3-3 beta and zeta. In vivo stoichiometric phosphorylation in brain at a Ser-Pro-Glu-Lys MOTIF. *J Biol.Chem.* **270**, 5706-5709 (1995).

169. Fu, H., Subramanian, R. R., & Masters, S. C. 14-3-3 proteins: structure, function, and regulation. *Annu.Rev.Pharmacol.Toxicol.* **40**, 617-647 (2000).

170. Boston, P. F., Jackson, P., & Thompson, R. J. Human 14-3-3 Protein - Radioimmunoassay, Tissue Distribution, and Cerebrospinal-Fluid Levels in Patients with Neurological Disorders. *Journal of Neurochemistry* **38**, 1475-1482 (1982).

171. Celis, J. E. *et al.* Comprehensive two-dimensional gel protein databases offer a global approach to the analysis of human cells: the transformed amnion cells (AMA) master database and its link to genome DNA sequence data. *Electrophoresis* **11**, 989-1071 (1990).

172. Leffers, H. *et al.* Molecular cloning and expression of the transformation sensitive epithelial marker stratifin. A member of a protein family that has been involved in the protein kinase C signalling pathway. *J Mol.Biol.* **231**, 982-998 (1993).

173. Fanger, G. R. *et al.* 14-3-3 proteins interact with specific MEK kinases. *J Biol.Chem.* **273**, 3476-3483 (1998).

174. Garcia-Guzman, M., Dolfi, F., Russello, M., & Vuori, K. Cell adhesion regulates the interaction between the docking protein p130(Cas) and the 14-3-3 proteins. *J Biol.Chem.* **274**, 5762-5768 (1999).

175. Freed, E., Symons, M., Macdonald, S. G., McCormick, F., & Ruggieri, R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science* **265**, 1713-1716 (1994).

176. Ichimura, T., Isobe, T., Okuyama, T., Yamauchi, T., & Fujisawa, H. Brain 14-3-3 protein is an activator protein that activates tryptophan 5-monooxygenase and tyrosine 3-monooxygenase in the presence of Ca2+,calmodulin-dependent protein kinase II. *FEBS Lett.* **219**, 79-82 (1987).

177. Ichimura, T. *et al.* Molecular cloning of cDNA coding for brain-specific 14-3-3 protein, a protein kinase-dependent activator of tyrosine and tryptophan hydroxylases. *Proc.Natl.Acad.Sci U.S.A* **85**, 7084-7088 (1988).

178. Xiao, B. *et al.* Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. *Nature* **376**, 188-191 (1995).

179. Liu, D. *et al.* Crystal structure of the zeta isoform of the 14-3-3 protein. *Nature* **376**, 191-194 (1995).

180. Yaffe, M. B. How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett.* **513**, 53-57 (2002).

181. Zeng, Y. *et al.* Replication checkpoint requires phosphorylation of the phosphatase Cdc25 by Cds1 or Chk1. *Nature* **395**, 507-510 (1998).

182. Aitken, A. Functional specificity in 14-3-3 isoform interactions through dimer formation and phosphorylation. Chromosome location of mammalian isoforms and variants. *Plant Mol.Biol.* **50**, 993-1010 (2002).

183. Kubala, M., Obsil, T., Obsilova, V., Lansky, Z., & Amler, E. Protein modeling combined with spectroscopic techniques: an attractive quick alternative to obtain structural information. *Physiol Res.* **53 Suppl 1**, S187-S197 (2004).

184. Aitken, A. *et al.* 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. *Trends Biochem.Sci* **17**, 498-501 (1992).

185. Toker, A., Ellis, C. A., Sellers, L. A., & Aitken, A. Protein kinase C inhibitor proteins. Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. *Eur.J Biochem.* **191**, 421-429 (1990).

186. Morgan, A. & Burgoyne, R. D. Exo1 and Exo2 proteins stimulate calcium-dependent exocytosis in permeabilized adrenal chromaffin cells. *Nature* **355**, 833-836 (1992).

187. Fu, H., Coburn, J., & Collier, R. J. The eukaryotic host factor that activates exoenzyme S of Pseudomonas aeruginosa is a member of the 14-3-3 protein family. *Proc.Natl.Acad.Sci U.S.A* **90**, 2320-2324 (1993).

188. Campbell, J. K. *et al.* Activation of the 43 kDa inositol polyphosphate 5-phosphatase by 14-3-3zeta. *Biochemistry* **36**, 15363-15370 (1997).

189. Hunter, T. Oncoprotein networks. *Cell* **88**, 333-346 (1997).

190. Furlanetto, R. W., Dey, B. R., Lopaczynski, W., & Nissley, S. P. 14-3-3 proteins interact with the insulin-like growth factor receptor but not the insulin receptor. *Biochem.J* **327 (Pt 3)**, 765-771 (1997).

191. Craparo, A., Freund, R., & Gustafson, T. A. 14-3-3 (epsilon) interacts with the insulin-like growth factor I receptor and insulin receptor substrate I in a phosphoserine-dependent manner. *J Biol.Chem.* **272**, 11663-11669 (1997).

192. Kino, T. *et al.* Protein 14-3-3sigma interacts with and favors cytoplasmic subcellular localization of the glucocorticoid receptor, acting as a negative regulator of the glucocorticoid signaling pathway. *J Biol.Chem.* **278**, 25651-25656 (2003).

193. Tazawa, H., Takahashi, S., & Zilliacus, J. Interaction of the parathyroid hormone receptor with the 14-3-3 protein. *Biochim.Biophys.Acta* **1620**, 32-38 (2003).

194. Fantl, W. J. *et al.* Activation of Raf-1 by 14-3-3 proteins. *Nature* **371**, 612-614 (1994).

195. Freed, E., Symons, M., Macdonald, S. G., McCormick, F., & Ruggieri, R. Binding of 14-3-3 proteins to the protein kinase Raf and effects on its activation. *Science* **265**, 1713-1716 (1994).

196. Zha, J., Harada, H., Yang, E., Jockel, J., & Korsmeyer, S. J. Serine phosphorylation of death agonist BAD in response to survival factor results in binding to 14-3-3 not BCL-X(L). *Cell* **87**, 619-628 (1996).

197. Reuther, G. W., Fu, H., Cripe, L. D., Collier, R. J., & Pendergast, A. M. Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science* **266**, 129-133 (1994).

198. Bonnefoy-Berard, N. *et al.* Inhibition of phosphatidylinositol 3-kinase activity by association with 14-3-3 proteins in T cells. *Proc.Natl.Acad.Sci U.S.A* **92**, 10142-10146 (1995).

199. Conklin, D. S., Galaktionov, K., & Beach, D. 14-3-3 proteins associate with cdc25 phosphatases. *Proc.Natl.Acad.Sci U.S.A* **92**, 7892-7896 (1995).

200. Zhang, S. H., Kobayashi, R., Graves, P. R., Piwnica-Worms, H., & Tonks, N. K. Serine phosphorylation-dependent association of the band 4.1-related protein-tyrosine phosphatase PTPH1 with 14-3-3beta protein. *J Biol.Chem.* **272**, 27281-27287 (1997).

201. Ogihara, T. *et al.* 14-3-3 protein binds to insulin receptor substrate-1, one of the binding sites of which is in the phosphotyrosine binding domain. *J Biol.Chem.* **272**, 25267-25274 (1997).

202. Garcia-Guzman, M., Dolfi, F., Russello, M., & Vuori, K. Cell adhesion regulates the interaction between the docking protein p130(Cas) and the 14-3-3 proteins. *J Biol.Chem.* **274**, 5762-5768 (1999).

203. Vincenz, C. & Dixit, V. M. 14-3-3 proteins associate with A20 in an isoform-specific manner and function both as chaperone and adapter molecules. *J Biol.Chem.* **271**, 20029-20034 (1996).

204. Pallas, D. C. *et al.* Association of polyomavirus middle tumor antigen with 14-3-3 proteins. *Science* **265**, 535-537 (1994).

205. Muslin, A. J., Tanner, J. W., Allen, P. M., & Shaw, A. S. Interaction of 14-3-3 with signaling proteins is mediated by the recognition of phosphoserine. *Cell* **84**, 889-897 (1996).

206. Yaffe, M. B. *et al.* The structural basis for 14-3-3:phosphopeptide binding specificity. *Cell* **91**, 961-971 (1997).

207. Masters, S. C., Pederson, K. J., Zhang, L., Barbieri, J. T., & Fu, H. Interaction of 14-3-3 with a nonphosphorylated protein ligand, exoenzyme S of Pseudomonas aeruginosa. *Biochemistry* **38**, 5216-5221 (1999).

208. Wang, B. *et al.* Isolation of high-affinity peptide antagonists of 14-3-3 proteins by phage display. *Biochemistry* **38**, 12499-12504 (1999).

209. Muslin, A. J. & Xing, H. 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal.* **12**, 703-709 (2000).

210. Seimiya, H. *et al.* Involvement of 14-3-3 proteins in nuclear localization of telomerase. *EMBO J* **19**, 2652-2661 (2000).

211. Tang, S. J., Suen, T. C., McInnes, R. R., & Buchwald, M. Association of the TLX-2 homeodomain and 14-3-3eta signaling proteins. *J Biol.Chem.* **273**, 25356-25363 (1998).

212. Jiang, K. *et al.* Regulation of Chk1 includes chromatin association and 14-3-3 binding following phosphorylation on Ser-345. *J Biol.Chem.* **278**, 25207-25217 (2003).

213. Nomura, M. *et al.* 14-3-3 Interacts directly with and negatively regulates pro-apoptotic Bax. *J Biol.Chem.* **278**, 2058-2065 (2003).

214. Kumagai, A. & Dunphy, W. G. Binding of 14-3-3 proteins and nuclear export control the intracellular localization of the mitotic inducer Cdc25. *Genes Dev.* **13**, 1067-1072 (1999).

215. Yang, J., Winkler, K., Yoshida, M., & Kornbluth, S. Maintenance of G2 arrest in the Xenopus oocyte: a role for 14-3-3-mediated inhibition of Cdc25 nuclear import. *EMBO J* **18**, 2174-2183 (1999).

216. Grozinger, C. M. & Schreiber, S. L. Regulation of histone deacetylase 4 and 5 and transcriptional activity by 14-3-3-dependent cellular localization. *Proc.Natl.Acad.Sci U.S.A* **97**, 7835-7840 (2000).

217. Brunet, A. *et al.* Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor. *Cell* **96**, 857-868 (1999).

218. Cahill, C. M. *et al.* Phosphatidylinositol 3-kinase signaling inhibits DAF-16 DNA binding and function via 14-3-3-dependent and 14-3-3-independent pathways. *J Biol.Chem.* **276**, 13402-13410 (2001).

219. Dhillon, A. S., Meikle, S., Yazici, Z., Eulitz, M., & Kolch, W. Regulation of Raf-1 activation and signalling by dephosphorylation. *EMBO J* **21**, 64-71 (2002).

220. Wang, Y. *et al.* The RAS effector RIN1 directly competes with RAF and is regulated by 14-3-3 proteins. *Mol.Cell Biol.* **22**, 916-926 (2002).

221. Ory, S., Zhou, M., Conrads, T. P., Veenstra, T. D., & Morrison, D. K. Protein phosphatase 2A positively regulates Ras signaling by dephosphorylating KSR1 and Raf-1 on critical 14-3-3 binding sites. *Curr.Biol.* **13**, 1356-1364 (2003).

222. Cavet, M. E., Lehoux, S., & Berk, B. C. 14-3-3beta is a p90 ribosomal S6 kinase (RSK) isoform 1-binding protein that negatively regulates RSK kinase activity. *J Biol.Chem.* **278**, 18376-18383 (2003).

223. Niu, J. *et al.* RGS3 interacts with 14-3-3 via the N-terminal region distinct from the RGS (regulator of G-protein signalling) domain. *Biochem.J* **365**, 677-684 (2002).

224. Benzing, T. *et al.* 14-3-3 interacts with regulator of G protein signaling proteins and modulates their activity. *J Biol.Chem.* **275**, 28167-28172 (2000).

225. O'Kelly, I., Butler, M. H., Zilberberg, N., & Goldstein, S. A. Forward transport. 14-3-3 binding overcomes retention in endoplasmic reticulum by dibasic signals. *Cell* **111**, 577-588 (2002).

226. Yuan, H., Michelsen, K., & Schwappach, B. 14-3-3 dimers probe the assembly status of multimeric membrane proteins. *Curr.Biol.* **13**, 638-646 (2003).

227. Moore, B. W. & McGregor, D. Chromotographic and electrophoretic fractionation of soluble proteins of brain and liver. *J Biol.Chem.* **240**, 1647-1653 (1965).

228. Green, A. J. Use of 14-3-3 in the diagnosis of Creutzfeldt-Jakob disease. *Biochem.Soc.Trans.* **30**, 382-386 (2002).

229. Colucci, M. *et al.* The 14-3-3 protein in multiple sclerosis: a marker of disease severity. *Mult.Scler.* **10**, 477-481 (2004).

230. Green, A. J. Cerebrospinal fluid brain-derived proteins in the diagnosis of Alzheimer's disease and Creutzfeldt-Jakob disease. *Neuropathol.Appl.Neurobiol.* **28**, 427-440 (2002).

231. Ghahary, A. *et al.* Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. *J Invest Dermatol.* **122**, 1188-1197 (2004).

Chapter 2

Extracellular 14-3-3 proteins: Potential contribution to airway tissue remodeling

2.1 Introduction:

Tissues rarely retain their structure without undergoing some form of modification. Inflammation, growth, aging and maturation are factors that contribute to changes generally recognized as tissue remodeling. Remodeling is a critical aspect of wound healing/repair in all organs representing a dynamic process aimed at maintaining equilibrium between matrix production and degradation[1,2]. In the airways, remodeling is a common consequence and central feature of many lung diseases, including asthma, chronic obstructive pulmonary disease and other disorders of lung matrix remodeling[3]. These conditions in spite of clinical distinctions, share a common paradigm of disease progression. In the context of injury, activated epithelial and inflammatory cells emit signals that provoke ingrowth and/or expansion of connective tissue elements which lead to persistent and at times permanent matrix reordering[3].

Asthma is an inflammatory disease of the airways, leading to progressive lung function impairment[4]. While inflammation has been a major focus of attention in asthma, parallel research on airway remodeling, an equally important pathophysiological feature of this disease, has received relatively less attention. Indeed, relevance of remodeling to disease pathogenesis remains controversial[5]. Tissue remodeling in asthma involves epithelial desquamation, mucus gland and airway smooth muscle (ASM) hypertrophy and hyperplasia as well as alteration in the general profile of extracellular matrix (ECM) proteins[6]. Collagen I, III and V, fibronectin, tenascin, versican, and laminin levels are increased[7]. In contrast, collagen IV and elastin appear to be decreased [8].

Matrix Metalloproteinases (MMPs) comprise a family of over 20 endopeptidases containing Zn^{2+} and Ca^{2+} ,capable of cleaving components of extracellular matrix[9]. MMPs represent a diverse group of proteolytic enzymes involved in ECM turnover and connective tissue remodeling during many physiological conditions including embryonic growth and development, uterine involution, bone growth and resorption, and wound healing[10]. Recently, it has been demonstrated that MMPs are likely to contribute to the pathogenesis of inflammation and airway remodeling in asthma[11]. MMP-1, -2, -3, -8 and –9, are detectable in asthmatic airways or the lung of asthmatic patients[11-14], with MMP-1 being mainly secreted by fibroblasts and activated monocytes/macrophages[15,16]. Although MMP-1 displays a rather limited proteolytic spectrum in the context of extracellular matrix, it is a major fibrillar collagen degrading protease[17]. It is therefore likely that in asthma, increased levels of MMP-1 is mainly associated with bronchial remodeling involving impaired cycles of degradation and repair. Moreover other catalytic activities of MMP-1 in the asthmatic airways, including its contribution to the insulin-like growth factor (IGF) axis[18,19], may play a role in airway remodeling. Evidence has shown that IGFs and IGF-binding proteins (IGF-BPs) are important regulators of airway smooth muscle (ASM) cells growth and proliferations[20]. The bioavailability of IGFs to exert their proliferative activity on smooth muscles depends on the presence of proteases that could cleave the high affinity-inhibitory IGF-BPs [21,22]. *Rajah et al* 1999, showed expression of immunoreactive MMP-1 to be dramatically increased in asthmatic airway smooth-muscle cells, when quantified using both visual and densitometric

analyses[23]. Further, MMP-1 functions as an important protease to degrade insulin-like growth factor binding proteins (IGF-BP)-2 and -3[14], thus increasing the availability of IGF-1. Taken together, the above observations provide strong evidence for the implication of IGFBP proteolysis by MMP-1 in the pathophysiology of asthmatic airway remodeling.

The regulation of MMPs expression is the result of complex interactions between various mediators and cells. Many proinflammatory cytokines shown to be increased in asthmatic airways, including monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-β_1 (TGF-β_1), also stimulate MMP-1 expression in different cell types[24,25]. In fibroblasts, however TGF-β_1 is widely assumed to exert inhibitory effects on MMP-1 gene expression[26]. Recently, our group isolated a novel MMP-1-inducing factor designated, keratinocyte-derived anti-fibrogenic factor (KDAF), from keratinocyte-conditioned medium (KCM) which was subsequently identified as the secreted form of stratifin, 14-3-3 σ[27].

To study the epithelial-mesenchymal communication and events leading to airway remodeling in asthma, I hypothesized that a lung-derived 14-3-3, is an important product of epithelial cells to enhance collagenase expression in lung fibroblasts. This signal may be an important factor to increase collagenase levels in asthma during ongoing inflammation and remodeling.

2.2 Materials and Methods:

Cell culture and Reagents: The human pulmonary type II alveolar epithelial cell line, A549, was purchased from American Type Culture Collection, Rockville, Md and seeded into 75-cm^2 flasks (Corning Inc), maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY), 4mmol/L L-glutamine, and 100 μg/ml penicillin/ streptomycin (Gibco) in a humidified 5% CO_2 atmosphere. The medium was replaced 24 hr before experiment with 1% FBS-DMEM, supplemented with 4mmol/L glutamine and 100 μg/ml penicillin/streptomycin. Conditioned medium was collected after 24 hr and subjected to lactate dehydrogenase (LDH) and Western blot analyses. Cells were harvested by brief treatment with 0.1% trypsin (Life Technologies Inc., Gaithersburg, MD) and 0.02 % ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (PH 7.4) and counted using trypan blue for cell viability.

HS-24, a human bronchial squamous carcinoma cell line (W. Ebert, Thoraxklinikum, Heidelberg-Rohrbach, Germany), was maintained in RPMI 1640 (American Type culture collection(ATCC), Manassas, USA) with 25 mM HEPES, pH 7.4, 2 mM glutamine, 100 μg/ml penicillin/streptomycin and 10% fetal bovine serum. The medium was replaced 24 hr before experiment with 1% FBS-RPMI supplemented with 2 mM glutamine, 100 μg/ml penicillin/streptomycin. Conditioned medium was collected as described above.

Dermal fibroblasts: Cultures of fibroblasts from human foreskin were established as described previously[28]. Briefly, punch biopsy samples were prepared from human foreskins in Dulbecco's modified Eagle medium (DMEM) with 10% fetal bovine serum. Biopsies were minced into small pieces of less than 0.5 mm in any dimension, washed with sterile medium (x6), and distributed into 60x15 mm Petri culture dishes (Corning Inc., Corning, NY) (4pieces per dish). A sterile glass coverslip was attached to the dish with a drop of sterile silicone grease to immobilize tissue fragments. To each dish was added DMEM and antibiotics (penicillin G sodium 100 U/ml, streptomycin sulfate 100 μg/ml, and amphotericin B 0.25 μg/ml; 3 ml) with 10% FBS and incubated at 37°C (water-jacketed, 95% humidity) in an atmosphere of 5% CO_2. The medium was replaced twice weekly. After 4 weeks of incubation, cells were removed from dishes following a brief (5-minute) treatment with 0.1% trypsin and 0.02% EDTA in phosphate buffered saline solution (PBS; pH 7.4) and transferred to 75 cm^2 culture flasks. Thereafter, once visual confluence was reached, the cells were subcultured 1:6 by trypsinization. Fibroblasts from passages 4-7 were used for this study.

Lung Fibroblasts: Cells of human diploid fibroblast strain IMR-90 was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Eagle Minimum essential medium (EMEM) (Gibco) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, FBS 10% in a humidified 5% CO_2 atmosphere.

Measurement of LDH in cell supernatants

The concentration of LDH was determined in supernatants of the cultured cells as a marker for cell lysis (Sigma diagnostic kit No.500, colorimetric, endpoint) using a colorimetric enzymatic method. Briefly nicotinamide adenine dinucleotide NAD^+ was reduced to NADH and H^+ by oxidation of lactate to pyruvate. In the second step the catalyst transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT (iodophenyl-nitrophenyl-phenyltetrazolium chloride) which in turn is reduced to formazan. The increase in the amount of enzyme activity in the medium correlates directly with the amount of formazan formed during a limited time period. Thus, the amount of color formed in the assay is proportional to the number of lysed cells. The absorbance of the samples was measured at 450 nm, using a microtiter plate reader. I used Free medium (DMEM) as negative control, and A549 or HS24 cell lysates obtained from each cell culture flask were used as positive control in proportion to the amount of condition-medium assayed (Figure 2.3).

Western blot analysis

Collected conditioned media (CM) were concentrated using a Centricon YM-3 filter device (30Kd cutoff) (Millipore Corporation, Beford, MA). Total volume used was adjusted to cell numbers of each culture flask. After concentration, total volumes in each centricon tube were adjusted to an equal volume using double-distilled water. Concentrated CM was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a 12% (wt/vol) acrylamide gel, and electrotransferred onto PVDF membranes (Millipore

Corporation). Active sites on the membranes were blocked in 5% skim milk powder in PBS 0.1% Tween 20 overnight. Immunoblotting was performed using 2μg/ml of goat anti-human 14-3-3 σ polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), or 2μg/ml of goat anti-human 14-3-3 α/β antibody which recognizes an N-terminal peptide of all isoforms of 14-3-3 proteins (Santa Cruz Biotechnology, Inc.). The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-goat IgG (Sigma, Saint Louis, MI) (1:2500 dilution). Immunoreactive proteins were then visualized using ECL plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, England).

Human recombinant stratifin (14-3-3σ) and 14-3-3α/β:

Human recombinant 14-3-3σ and 14-3-3 α/β were prepared as described previously[27]. Briefly, cDNA of 14-3-3σ from human keratinocytes and cDNA of 14-3-3α/β from human fibroblasts, respectively, were cloned into pGEX-6P-1 expression vector (Amersham/ Pharmacia Biotech) and transformed into protein expressing bacteria, BL-21 (DE3)(Novagene). A single positive clone was grown in 100 ml of LB medium containing 50 μg/ml of ampicillin for 4-6 hr at 29°C until an OD_{600nm} of 0.4-0.6 was reached. Bacteria were then diluted to 1: 10 with LB medium plus 0.1 mM IPTG for 12 hr. To purify the protein, bacteria were centrifuged and lysed with 50mM Tris-HCL (PH 7.4) containing 10mM EDTA, 5mM EGTA, protease cocktail (Sigma), 1% Triton X-100, and 0.5% IGEPAL CA630. Cell lysate was passed through a glutathione sepharose-4B affinity column and subsequently washed with PBS containing 0.1% Triton X-100 until

an OD_{280nm} reached zero. GST-fused 14-3-3σ was digested using PreScission protease according to manufacturer's procedure (Amersham/ Pharmacia Biotech). GST-free 14-3-3σ and 14-3-3 α/β were then eluted, dialysed against PBS and then concentrated with Centricon (Millipore). The sequence of proteins were validated on a Bruker REFLEX III mass spectrometer (Bremen/Leipzig, Germany) using MALDI in positive ion mode.

Treatment of primary dermal and lung (IMR-90) fibroblasts:

Fibroblasts were seeded into 60X15-mm petri dishes (Corning Inc.) For each experiment, confluent fibroblast were rinsed with phosphate-buffer saline (PBS) before fresh medium (DMEM or MEME) supplemented with 10% FBS were added. At this time, fibroblasts were treated with either14-3-3σ (2.5 ug/ml), 14-3-3β (1, 1.5, 2.0, 2.5, 3.0 ug/ml), TGF-β_1(150 pg/ml) or conditioned medium collected from A549 (LCM) respectively.

Epithelial cells and fibroblasts were cultured in the upper and lower chambers of the co-culture system, as previously described[29]. Briefly fibroblasts were cultured in the lower chamber of the co-culture system until confluent. Epithelial cells were separately cultured on six-well plates and subcultured upon confluence, into the top chamber of the co-culture system. Total RNA was extracted from fibroblasts grown in the lower chamber and the expression of collagenase mRNA was evaluated by Northern analysis. Fibroblasts grown in a fibroblast/fibroblast co-culture system were used as controls.

RNA isolation and Northern blot analysis:

Fibroblasts were harvested with 400 µl of 4M guanidium isothiocynate (GITC) solution and total RNA from each group was isolated by the acid-guanidium-phenol chloroform method [30]. Total RNA from each individual fibroblast culture was then separated by electrophoresis (10 µg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, California). The blots were baked for 2hr at 80°C under vacuum and prehybridized for 4hr at 45°C in a prehybridization solution. Hybridization was performed at 45°C in the same solution, using collagenase cDNA probes. The cDNA probe for collagenase was obtained from the American Type Culture Collection (Rockville, MD) and labelled with P-α^{32}-dCTP by nick translation. The filters were washed initially at room temperature with 2X sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1hr and finally washed for 20 min at 65°C in 0.1X sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at -80°C in the presence of intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry.

Immunoprecipitation of 14-3-3

Confluent lung epithelial cells (A549; ATCC), were kept in (DMEM, GIBCO) containing glutamine (2mM), non-essential amino acids (100 µM), antibiotics (final concentration: Penicillin 100 units/ml, Streptomycin 100 µg/ml; Sigma),

and 1% (FBS, GIBCO) for 24 hours. Culture medium was collected and either used directly or subjected to immunodepletion of 14-3-3. Immunoprecipitation was performed using polyclonal antibody (IgG) against purified 14-3-3 raised in rabbit [customized by Washington Biotechnology, Inc. (Baltimore, MD)] that recognizes all isoforms of 14-3-3 proteins described in our previous study [27]. Antibody at final concentration of 10 μg / ml was added to lung conditioned medium (LCM)] and incubated at 4°C for overnight on the rotator. The next day, 50 μl of protein A/G-agarose (Santa Cruz) was added and tube was kept rotating at 4°C for 2 hours. Tube was centrifuged at 4°C, 1000 RPM for 2-3 min. Supernatant was collected and used to treat lung fibroblasts.

Statistical analysis:

Autoradiograms of Northern analysis were quantified with densitometry and data expressed as a relative OD value. The statistical significance of differences in collagenase mRNA expression between treated and untreated dermal fibroblasts was determined using Student unpaired two-tailed *t*-test; p values< 0.05 were considered significant.

2.3 RESULTS

2.3.1: 14-3-3 proteins, but not 14-3-3σ, are present in A549 and HS24 epithelial cells supernatant

To study the presence of 14-3-3 proteins in conditioned media (CM) of A549 and HS24 cell lines, concentrated CM was subjected to Western blot analysis as described in materials and methods. Equally concentrated non-conditioned media supplemented with 1% FBS (1% NCM) were used as a control. As shown in figure 2.1, A & C when blotted with an antibody that can recognize all isoforms of 14-3-3 proteins, there was measurable amount of 14-3-3 protein in A549 and HS24 CM (n=4). Further SDS gels were blotted and probed for the presence of the σ isoform in separate set of experiments. As seen in figure 2.1, B & D, no detectable 14-3-3σ isoform was found in A549 and HS24 CM. Moreover, the absence of 14-3-3σ in both A549 (lung epithelial) conditioned medium (LCM) and A549 cell lysate (CL) was confirmed using isoform specific polyclonal antibodies raised against entire length of 14-3-3 peptide (figure 2.2). In a similar set of experiments we observed the presence of four other 14-3-3 isoforms γ, ε, η, ζ in CL and LCM of A549 cell line (figure 2.2).

To ascertain that measurable extra cellular 14-3-3 was not the result of cell lysis, LDH assay was performed on every batch of conditioned media prior to Western analysis (figure 2.3)

As shown in figure 2.3, A and Figure 2.3, B, LDH analysis of A549 and HS24 CM did not contain significant LDH enzyme, as compared to total LDH released

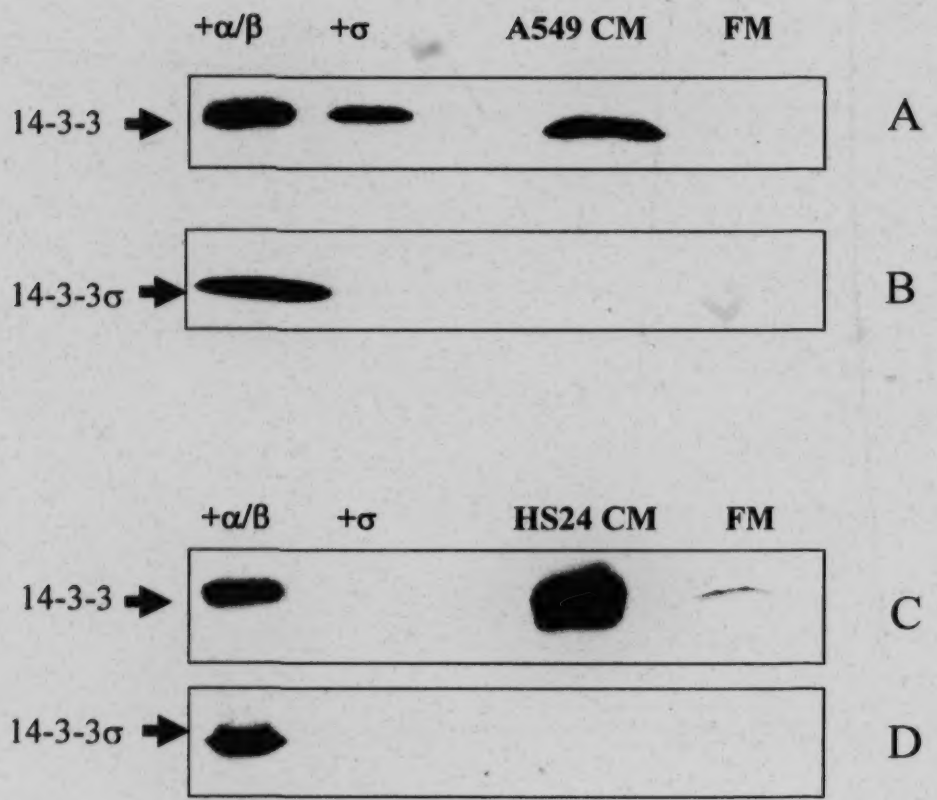


Figure 2.1: A, C Western blot analysis of A549 and HS 24 CM for detection of all isoforms of 14-3-3 (n = 4 separate experiments). B, D: Westernblot analysis of A549 and HS 24 CM for detection of σ isoforms of 14-3-3 (n = 4). +α/β: Positive control (recombinant 14-3-3 α/β), +σ: Positive control (recombinant 14-3-3σ), FM: free medium

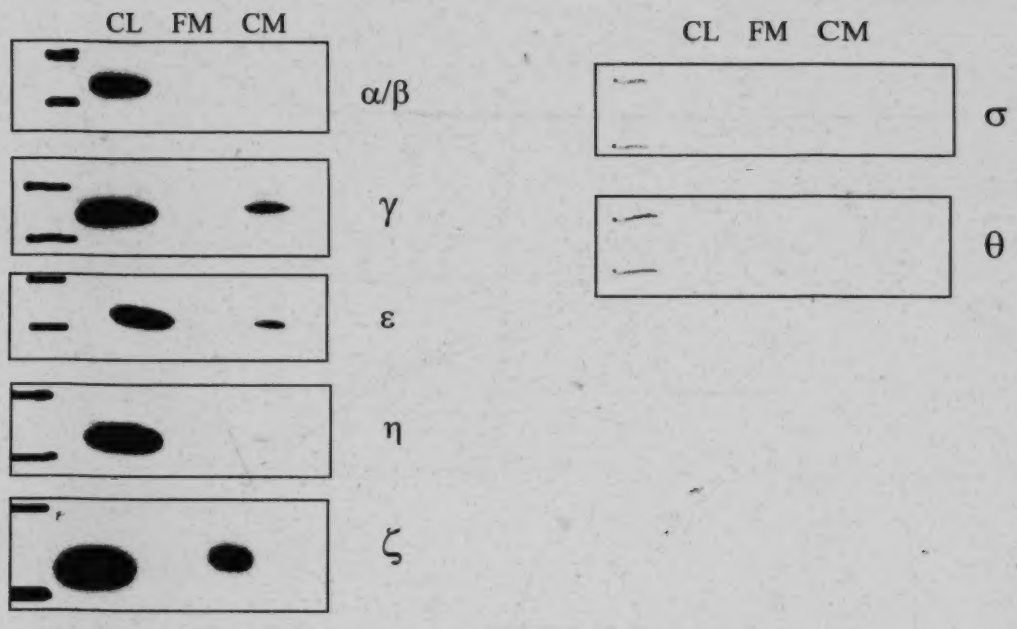
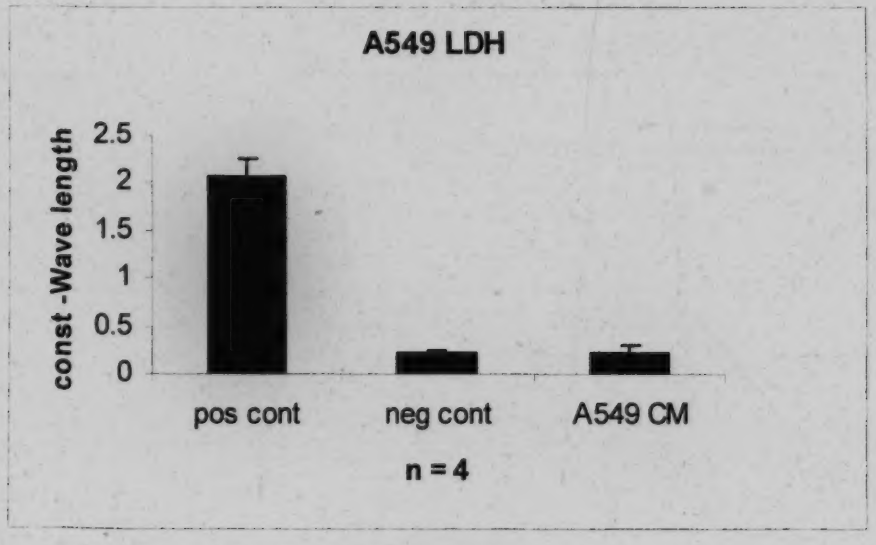
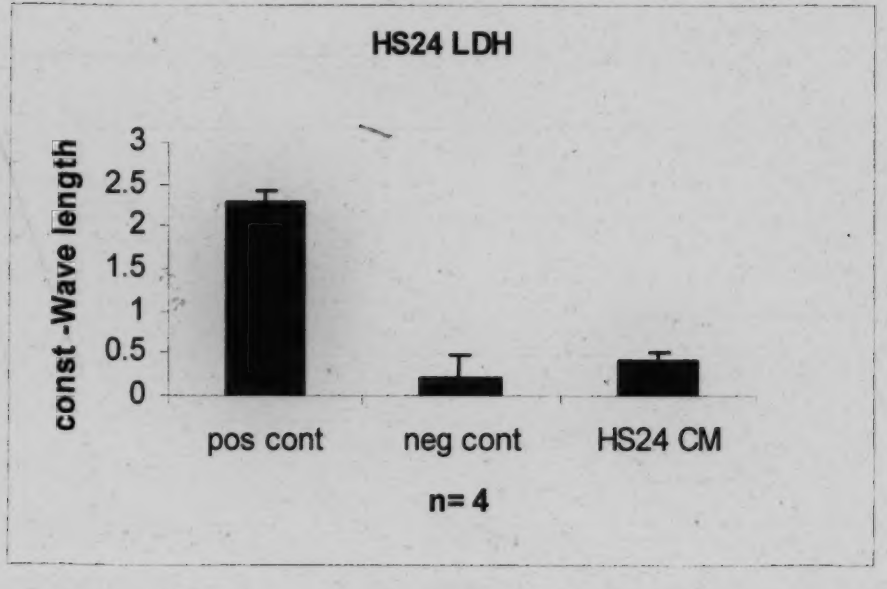


Figure 2.2: Western blot analysis of LCM for detection of isoforms (α/β, γ, ε, η, ζ, σ, θ) of 14-3-3 (n =1). CL: A549 cell lysate, FM: free (low FBS) medium, CM: conditioned medium of A549 cells following 24 hour incubation in low FBS medium.



A



B

Figure 2.3, A & B: LDH analysis of A549 and HS24 CM (n = 4). Post cont: Positive control, neg cont: negative control.

from each flask of cells (positive control), (LDH results are shown as percentage of positive control).

2.3.2 Lung epithelial cell line (A549) conditioned medium (LCM) induces MMP-1 in dermal fibroblasts:

As discussed earlier, our group[27] has previously shown that a releasable form of (14-3-3σ) from keratinocytes (KDAF), stimulate collagenase production in dermal fibroblasts. To examine the effect of LCM on induction of collagenase in dermal fibroblasts, the MMP-1 mRNA expression in dermal fibroblasts was studied, following treatment with LCM. As shown in figure 2.4, primary dermal fibroblasts were treated with either medium alone (Fig 2.4,A, lane 1-2), purified recombinant 14-3-3σ (KDAF) (2.5 μg/ml) (Fig 2.4, A, lanes3-4), LCM for 24hr. Expression of collagenase (MMP-1) mRNA was evaluated using Northern analysis as described in materials and methods. To rule out possible stimulatory effects of the fetal bovine serum (FBS) used in culture medium on MMP-1 expression, control (free medium (FM)) and the recombinant 14-3-3σ treatments were supplemented with either 10% or 1% FBS respectively. LCM contained 1% FBS. Figure 2.4 represents three separate experiments (n=3).

My data showed that treatment of primary dermal fibroblasts with LCM significantly increased MMP-1 mRNA expression (figure 2.4, A, lane5). Densitometry data from three separate experiments revealed highly significant differences in collagenase levels between controls, 14-3-3 σ and LCM treatments (**$p<0.0001$, *** $p<0.0005$, n=3) (Figure 2.4, C). Total RNA loading control was determined by 18S ribosomal RNA of the same blot (figure 2.4, B).

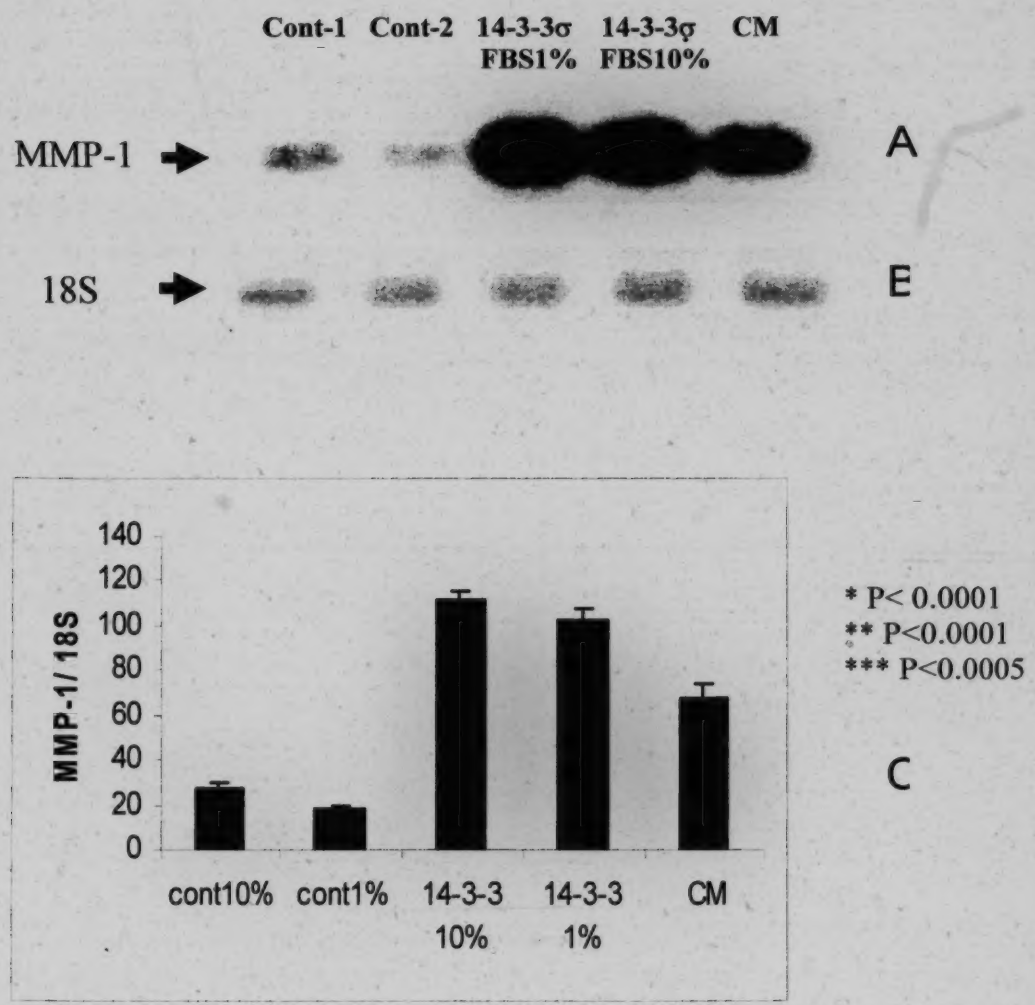


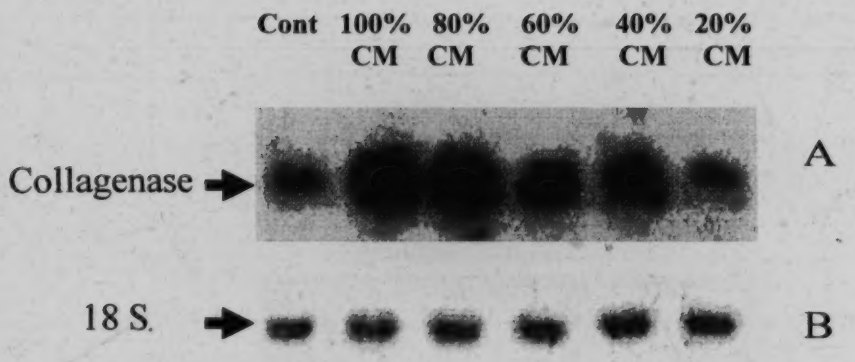
Figure 2.4; A: Northern blot analysis for detection of MMP-1 mRNA expression in dermal fibroblasts treated as mentioned in panel A. Panels A and B represent three separate experiments(n =3). B: Loading control with 18S ribosomal RNA. C: Student t-test analysis on densitometry data (MMP-1/18S RNA) from three independent experiments.

In a similar set of experiments I showed that LCM induced MMP-1 expression in a dose dependent manner (figure 2.5, n=3).

2.3.3 14-3-3 α/β, but not σ, induces MMP-1 mRNA expression in human lung fibroblasts

Lung (IMR 90) fibroblasts and primary dermal fibroblasts were treated with purified recombinant 14-3-3 σ, 14-3-3 α/β or LCM for 24hr. Total RNA was extracted from fibroblasts and the expression of collagenase (MMP-1) mRNA was evaluated by Northern analysis. MMP-1 mRNA expression was significantly increased in lung fibroblasts upon treatment with LCM (** $p<0.01$). In contrast to dermal fibroblasts, lung fibroblast MMP-1 mRNA expression was unaffected following treatment with 14-3-3σ. Interestingly, MMP-1 expression was significantly increased (*$p<0.01$) following treatment with 14-3-3α/β isoform. Total RNA loading control was determined by 18S ribosomal expression RNA on the same blot (figure 2.6, A), figure represents three different experiments (n = 3).

My results of MMP-1 mRNA expression showed that the lung fibroblast cell line, IMR 90 appeared to be unresponsive to 14-3-3σ but responded to α/β isoform treatment. This finding corresponds well with my earlier results, which showed no detectable 14-3-3σ isoform in lung epithelial cell supernatants.



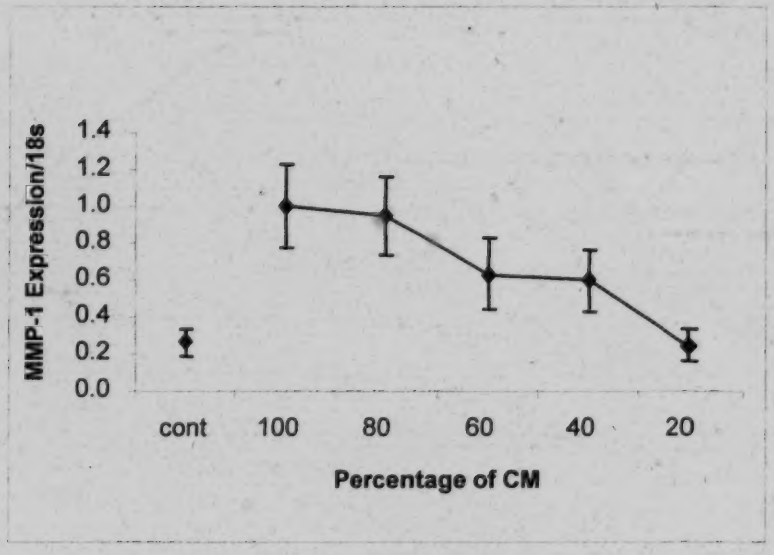


Figure 2.5; A, B and C: Northern blot analysis for detection of MMP-1 mRNA expression in dermal fibroblasts treated with different concentration of CM (n=3). B: Loading control with 18S ribosomal RNA.

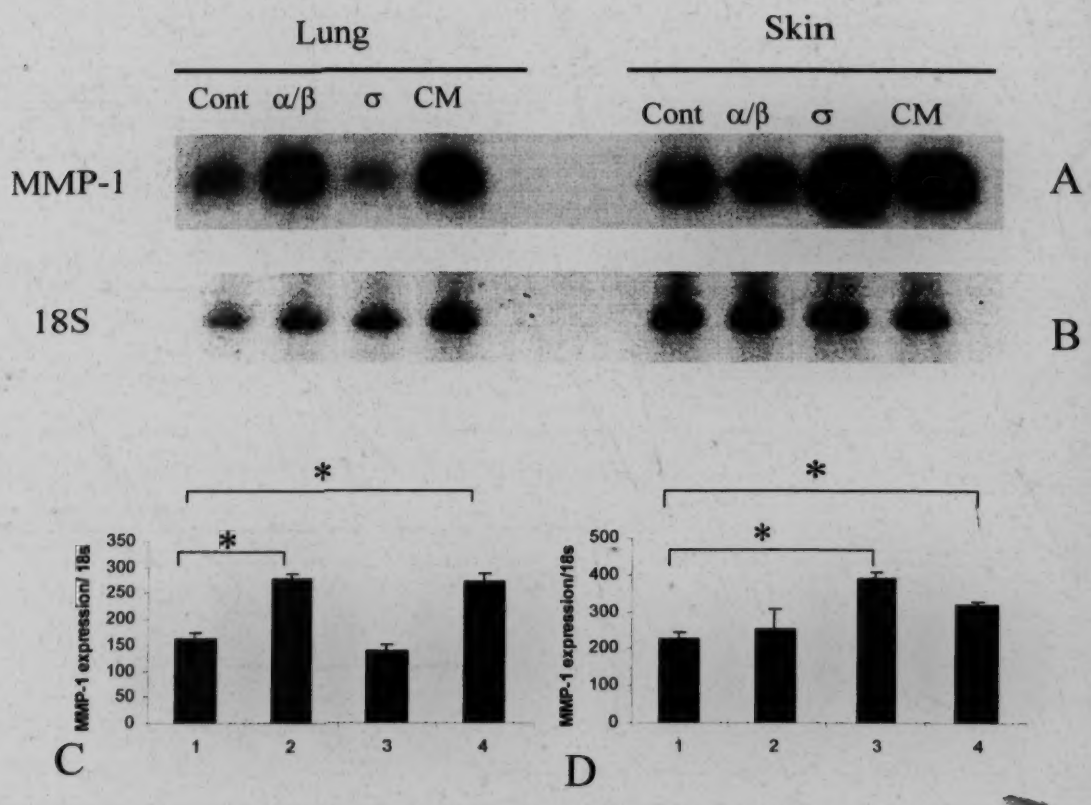


Figure 2.6, A: Northernblot analysis for detection of MMP-1 mRNA expression in dermal and lung fibroblasts treated as mentioned on each lane (n =3). B: Loading control with 18S ribosomal RNA. C, D: Student t-test analysis on densitometry data from three independent experiments. Fibroblasts treated as followed; lane 1: control, lane2: treated with 14-3-3 a/b, lane3: treated with 14-3-3 s, lane4: treated with CM. *P< 0.01

2.3.4 Immunodepletion of 14-3-3 decreases the effect of LCM in inducing MMP-1 mRNA expression

In this experiment, we examined and confirmed our previous observation that extracellular 14-3-3 proteins are responsible in major part for increase in MMP-1 mRNA expression in lung fibroblasts upon treatment with conditioned medium. All isoforms of 14-3-3 protein in LCM was immunoprecipitated (IP-L) using a rabbit polyclonal antibody against 14-3-3 proteins as described above. Lung fibroblasts (IMR 90) were treated with LCM, IP-L or medium alone. Total RNA from fibroblasts was extracted after 24hr and subjected to Northern analysis for expression of MMP-1. To correct for RNA loading, the signals for collagenase mRNA expression and 18S rRNA were determined by densitometry and the ratio of collagenase/18S was calculated. As shown in figure 2.7, and in accordance to our previous results, treatment of LCM increased MMP-1 mRNA expression in lung fibroblasts. However immunodepletion of all isoforms of 14-3-3 protein from LCM significantly decreased MMP-1 mRNA expression (figure 2.7, A, IP-L) (n=3). Taken together these results showed that the increase in MMP-1 mRNA level is in major part due to the presence of the extracellular form of 14-3-3 proteins as depletion of media of these proteins significantly decrease collagenase mRNA expression.

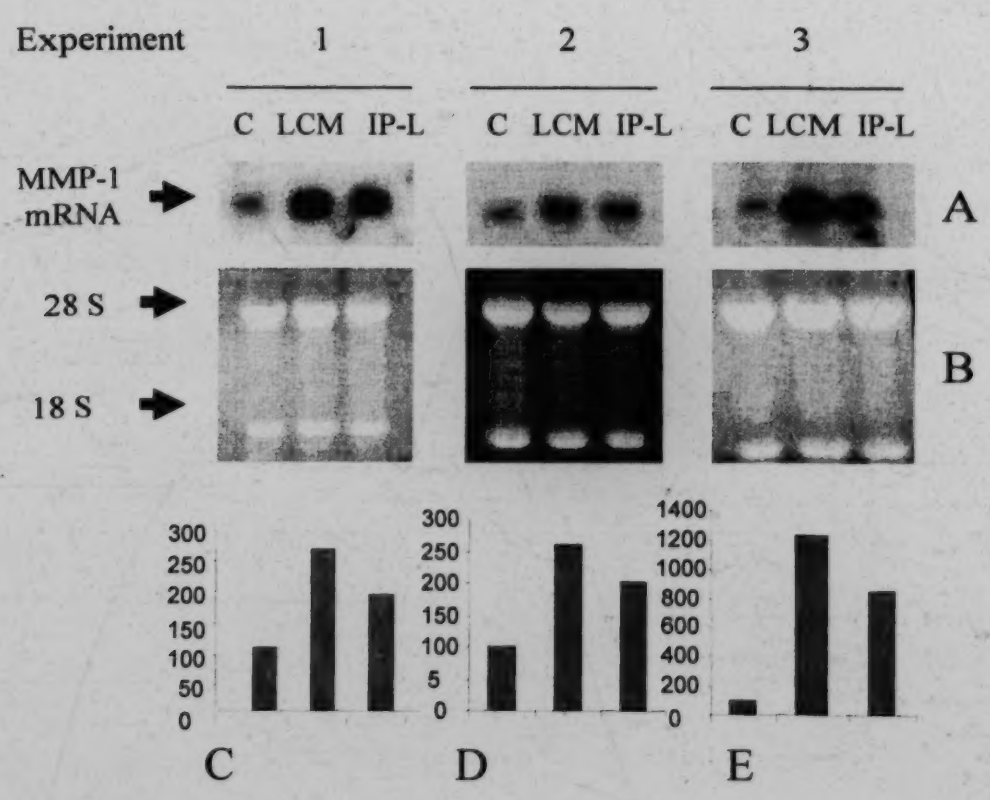


Figure 2.7; A: Northernblot analysis for detection of MMP-1 mRNA expression in dermal fibroblasts treated as mentioned on each lane (C: control, LCM: lung conditioned medium, IP-L : 14-3-3 immunodepleted LCM (n =3). B: Loading control with 18S and 28S ribosomal RNA. C, D, E : Densitometry data on percentage change of MMP-1 mRNA. Individual experiments shown due to large variability. This figure was generated by Dr. R.T Kilani

2.3.5 Lung epithelial Cells (A549) increase collagenase expression in human lung fibroblasts

To study the direct effect of epithelial cells rather than LCM, on inducing MMP-1 expression, we established an epithelial cell-fibroblasts co-culture system as described above. Lung epithelial cells and fibroblasts were grown in the upper and lower chambers of this system, respectively. Total RNA was extracted from fibroblasts in the lower chamber and the expression of collagenase (MMP-1) mRNA evaluated by Northern blot analysis. Lung fibroblasts (IMR 90) were also grown in the lower chamber in the presence of recombinant 14-3-3α/β or in a fibroblast/fibroblast co-culture system as controls (figure 2.8, A), figure represents three separate experiments (n=3). Densitometry results showed a very significant increase (**$p<0.0001$) in collagenase mRNA expression of fibroblasts grown in the epithelial/fibroblast co-culture system. No detectable collagenase mRNA was found in fibroblasts grown in the fibroblast-fibroblast co-culture system.

2.3.6 14-3-3α/β increases MMP-1 mRNA expression in the presence of MMP-1 inhibitory factor, TGF-β_1

The stimulatory effect of 14-3-3α/β on MMP-1 expression was further studied in the presence of the MMP-1 inhibitory cytokine, TGF-β_1. Lung fibroblasts (IMR90) were treated with 14-3-3 β (2.5 μg/ml) in the presence or absence of TGF-β_1 (150 pg/ml) or LCM and TGF-β_1 (150 pg/ml) for 24hr. Total RNA was

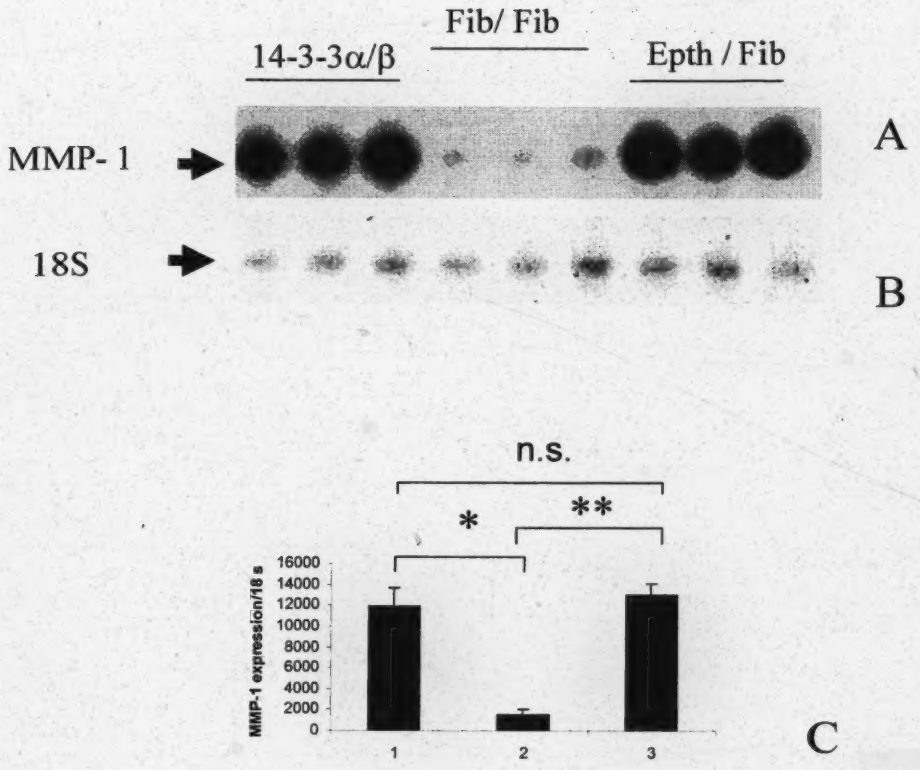


Figure 2.8; A: Northern blot analysis for detection of MMP-1 mRNA expression in lung fibroblasts in the co-culture system, treated with 14-3-3α/β or co-cultured with fibroblasts or epithelial cells (n =3). B: Loading control with 18S ribosomal RNA. C: Student t-test analysis on densitometry data (1: mean density lanes (1-3) of panel A, 2: mean density of lanes (4-6) of panel A, 3: mean density of lanes (7-9) of panel A). *P <0.001,**P<0.0001, n.s. = not significant.

extracted from fibroblasts and the expression of collagenase (MMP-1) mRNA was evaluated by Northern analysis (figure 2.9,A)

Densitometry results show that 14-3-3 α/β considerably increased MMP-1 mRNA expression in the presence or absence of TGF-β_1. Moreover, the stimulatory effect of LCM on MMP-1 expression was preserved with TGF-β_1 treatment (figure 2.9, C).

In addition, we compared the dose dependent effect of 14-3-3β on lung fibroblasts in the presence or absence of TGF-β_1 (figure 2.10, A, C). Lung fibroblasts (IMR90) were treated with a range of concentrations of purified recombinant 14-3-3α/β (0, 1, 1.5, 2, 2.5, 3 μg/ml) with or without TGF-β_1 (150 pg/ml) for 24hr. Total RNA was extracted from fibroblasts and MMP-1 mRNA expression was measured by Northern analysis.

Consistent with our previous observation in dermal fibroblasts [27] treatment of lung fibroblasts with recombinant 14-3-3α/β markedly increased MMP-1 expression in a dose-dependent manner (figure 2.10, A). The inhibitory role of TGF-β_1 on MMP-1 expression, however did not appear to decrease the 14-3-3α/β effect markedly (figure 2.10, C), suggesting a potent collagenase-stimulatory role for the extracellular form of 14-3-3α/β that may override the inhibitory effect of TGF-β_1.

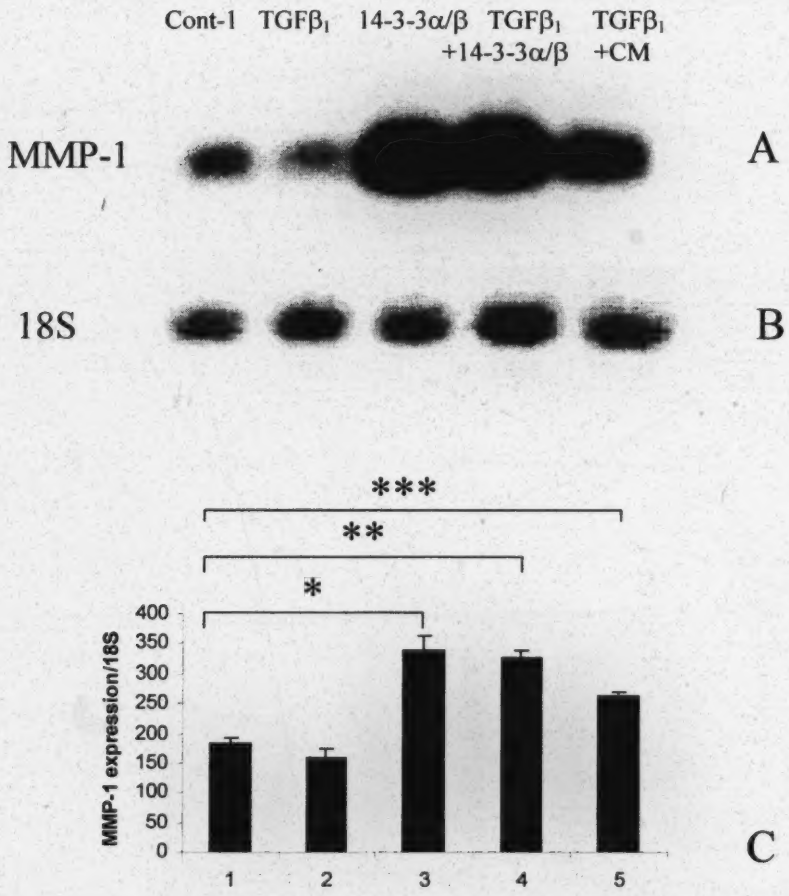


Figure 2.9 ;A: Northern blot analysis for detection of MMP-1 mRNA expression in lung fibroblasts treated as mentioned on each lane , TGF-β_1 150 pg/ml, 14-3-3 α/β(2.5μg/ml)(n =3). B: Loading control with 18S ribosomal RNA. C: Student t-test analysis on densitometry data from three independent experiments showing MMP-1/ 18s RNA. *P = 0.001, **P<0.01, *** P = 0.01

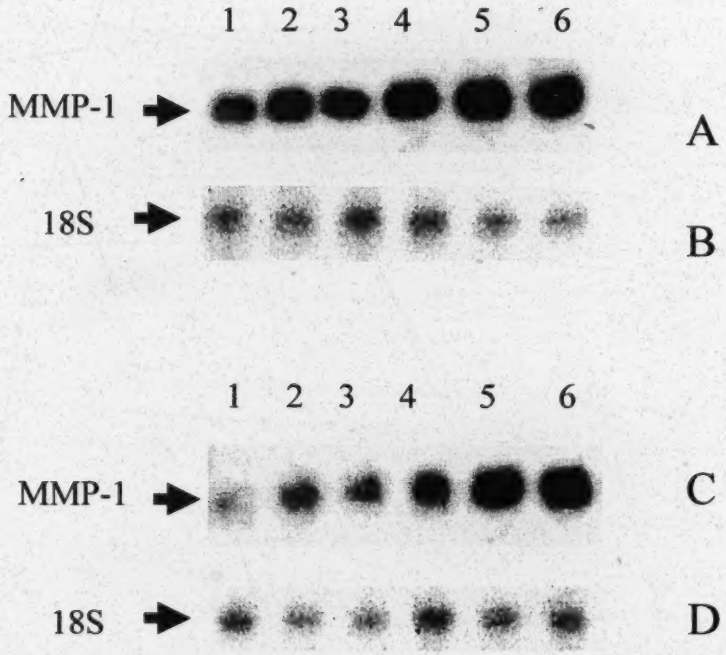


Figure 2.10; A: Northern blot analysis for detection of MMP-1 mRNA expression in lung fibroblasts treated with 14-3-3 α/β (0, 1, 1.5, 2, 2.5, 3 μg/ml) lane 1-6 respectively (n =2). B: Loading control with 18S rRNA. C: Northern blot analysis for detection of MMP-1 mRNA expression in dermal fibroblasts treated with TGF-β1 (150 pg/ml) and 14-3-3 α/β (0, 1, 1.5, 2, 2.5, 3 μg/ml), figure represents two different experiments. D: Loading control with 18S rRNA

2.5 Discussion:

Since the discovery of 14-3-3 proteins in 1967[31], a number of important biological activities have been attributed to the various isoforms of this family of proteins. In mammals, there are seven distinct isoforms (β, γ, ε, ζ, τ, η and σ), [32]. It is now known that the two isoforms initially described as α and δ represent the phosphorylated forms of β and ζ [33]. Eukaryotic cells express 14-3-3 proteins[34] which bind a multitude of functionally diverse ligands including over 50 signalling proteins[35], including kinases, phosphatases, and transmembrane receptors[36-40]. Through these interactions, 14-3-3 proteins participate in the regulation of a range of biological processes, including neuronal development, cell growth control and cell cycling[35,41]. Thus far, studies on 14-3-3 proteins have characterized their functions as intracellular proteins. The extracellular form of 14-3-3 can be detected in cerebrospinal fluid, and has lately been used as a diagnostic marker for prion diseases such as Creutzfeldt-Jakob disease and other neurological disorders[42,43]. However, the biological function of extracellular 14-3-3 proteins has only recently been explored[27]. These studies have identified the extracellular form of 14-3-3σ from keratinocytes as a collagenase (MMP-1) stimulating factor in skin fibroblasts[27].

Matrix metalloproteinases (MMPs) are involved in the various degradation of extracellular matrix scaffold proteins which enable tissue remodeling. MMP activity is regulated at three well characterized levels; gene transcription, post transcriptional activation of zymogens, and interactions of secreted MMPs with

tissue inhibitors of metalloproteinases (TIMPs)[44]. However, it is widely known that the main regulatory level for all MMPs except for MMP-2, occurs at the transcription level[45]. Transcriptional activation can be modulated by a variety of cytokines, growth factors and hormones including interleukin-1β (IL-1β), IL-6, tumor necrosis factor (TNF), epidermal growth factor (EGF), platelet-derived growth factor (PDGF), as well as IL-4, TGF-β_1 and corticosteroids. The impact of TGF-β_1 on MMP expression is cell-specific[46]. In human fibroblasts as mentioned earlier TGF-β_1 inhibits MMP-1 gene expression[47].

In this study, I tested the hypothesis that extracellular 14-3-3 proteins released from lung epithelial cells represent major collagenase (MMP-1)-inducing factor in lung fibroblasts. My data show for the first time that lung epithelial cell (A549) conditioned medium (LCM) has a potent stimulatory effect on MMP-1 both in primary dermal fibroblasts (dose dependent) and lung fibroblasts (IMR-90). By Western blot analysis, I identified the presence of 14-3-3 proteins but not the 14-3-3σ, in LCM as well as in bronchial epithelial cell (HS24) conditioned medium. Further, we have demonstrated that depleting of LCM from 14-3-3 proteins will significantly decrease the collagenase stimulatory affect of LCM. In addition, purified recombinant 14-3-3α/β recapitulated the LCM effect on induction of MMP-1 expression in lung fibroblasts. Interestingly, unlike previous observations in the skin[27], 14-3-3σ had no stimulatory effect on expression of MMP-1 in lung fibroblasts (figure 2.6). These findings further support my previous observation of the lack of 14-3-3σ in LCM. Taken together,

my data suggest that the capacity of 14-3-3 proteins to induce collagenase in different tissues may be isoform specific. Thus, I anticipate while 14-3-3σ may exert its effect in the skin, particularly in wound healing, other members of 14-3-3 family present in LCM (α/β,ε,η,γ and ζ) may be involved in inducing collagenase in the lung. However the direct effect of recombinant 14-3-3 ε,η,γ and ζ on MMP-1 expression on lung fibroblasts was not examined in the present study and is the subject of a future study.

The epithelial-mesenchymal communication is essential to physiological lung morphogenesis during embryonic development[48]. In asthma, it is widely assumed that similar growth factors, cytokines and other signalling molecules implicated in branching morphogenesis contribute to chronic changes in remodeled airways (i.e., reactivation of the epithelial-mesenchymal trophic unit (EMTU))[49]. In addition to various proinflammatory mediator released from injured epithelial cells in activated EMTU, these cells also release a number of profibrogenic factors including members of the TGF-β superfamily[50,51].

TGF-β is a family of multifunctional cytokines designated as TGF-β_1, TGF-β_2 and TGF-β_3. TGF-β_1 is a potent profibrotic factor which induces the synthesis of different ECM components including collagen type I and type III, fibronectin, tenascin and proteoglycans from fibroblasts[52]. TGF-β_1 further decreases the synthesis of ECM degrading enzymes such as stromelysin and collagenase (MMP-1) from fibroblasts and increases the production of inhibitors of these enzymes including plasminogen activator inhibitor type-1 (PAI-1) and tissue

inhibitor of metalloproteinase-1 (TIMP-1). Taken together TGF-β_1 is a factor with a profound impact on lung function, very likely involved in processes leading to airway remodeling in asthma[53]. Our data with lung fibroblast (IMR90) further confirmed the inhibitory effect of TGF-β_1 on expression of MMP-1 at transcriptional level (figure 2.9). However, we have shown that at least in regards to collagenase production, the TGF-β_1 collagenase-inhibitory effect at 150 pg/ml is suppressed by LCM. Moreover we showed that 14-3-3 α/β also mimicked the LCM effect on MMP-1 production; treatment with increasing doses (0, 1.0, 1.5, 2.0, 2.5, 3.0 ug/ml) of recombinant α/β isoform significantly induced collagenase expression in presence and absence of high doses (150 pg/ml) of TGF-β_1. These observations strongly suggest a potent collagenase (MMP-1) stimulatory role for lung epithelial cell- derived 14-3-3 proteins.

Our understanding of asthma pathophysiology has considerably changed over the past several decades. Today airway remodeling, a feature once thought to be solely associated with chronic inflammation, is thought to be present early in the course of the disease despite continuous anti-inflammatory treatment[54]. Many investigators have proposed excessive transcription of matrix metalloproteinases to be a key factor contributing to airway remodeling in asthma [14]. It is imperative, therefore to understand other regulatory factors than proinflammatory cytokines, responsible for over expression of MMPs, even in absence of inflammation. Based on the data of this study we suggest that the releasable α/β isoform of 14-3-3 proteins from lung epithelial cells represent a potent MMP-1-inducing factor in lung fibroblasts. The collagenase stimulating role of

extracellular 14-3-3 proteins, being non inflammatory mediators, further underscores the critical role of epithelial-mesenchymal communication in ECM modulation and pathophysiology of airway remodeling.

2.6 Reference List

1. Cotran R. Robbins Pathologic Basis of Disease in *Inflammation and repair* 39-87. Philadelphia: WB Saunders (2004).

2. Wilson JW, Bousquet J, Rak S, & Pauwels RA. Airway remodeling in *Airway remodeling* 1-2. Marcel Dekker, Inc, (2004).

3. Chapman, H. A. Disorders of lung matrix remodeling. *J Clin.Invest* **113**, 148-157 (2004).

4. Hilman B. NHLBI guidlines for diagnosis and management of asthma. *NIH publication* (2004).

5. Davies, D. E., Wicks, J., Powell, R. M., Puddicombe, S. M., & Holgate, S. T. Airway remodeling in asthma: new insights. *J.Allergy Clin.Immunol.* **111**, 215-225 (2003).

6. Lazaar, A. L. & Panettieri, R. A., Jr. Is airway remodeling clinically relevant in asthma? *Am.J.Med.* **115**, 652-659 (2003).

7. Laitinen, L. A. *et al.* Bronchial biopsy findings in intermittent or "early" asthma. *J.Allergy Clin.Immunol.* **98**, S3-S6 (1996).

8. Bousquet, J. *et al.* Asthma: a disease remodeling the airways. *Allergy* **47**, 3-11 (1992).

9. Ye, S. & Henney, A. M. Detecting polymorphisms in MMP genes. *Methods Mol.Biol.* **151**, 367-375 (2001).

10. Nagase, H. & Woessner, J. F., Jr. Matrix metalloproteinases. *J Biol.Chem.* **274**, 21491-21494 (1999).

11. Cataldo, D. *et al.* MMP-2- and MMP-9-linked gelatinolytic activity in the sputum from patients with asthma and chronic obstructive pulmonary disease. *Int.Arch Allergy Immunol.* **123**, 259-267 (2000).

12. Dahlen, B., Shute, J., & Howarth, P. Immunohistochemical localisation of the matrix metalloproteinases MMP-3 and MMP-9 within the airways in asthma. *Thorax* **54**, 590-596 (1999).

13. Mautino, G., Henriquet, C., Jaffuel, D., Bousquet, J., & Capony, F. Tissue inhibitor of metalloproteinase-1 levels in bronchoalveolar lavage fluid from asthmatic subjects. *Am.J.Respir.Crit Care Med.* **160**, 324-330 (1999).

14. Cataldo, D. D. *et al.* Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases mRNA transcripts in the bronchial secretions of asthmatics. *Lab Invest* **84**, 418-424 (2004).

15. Emonard H, Calle A, Grimaud JA, & et al. Interactions between fibroblasts and a reconstituted basement membrane matrix. *J Invest Dermatol* 156-163 (2004).

16. Finlay, G. A. *et al.* Matrix metalloproteinase expression and production by alveolar macrophages in emphysema. *Am.J Respir.Crit Care Med.* **156**, 240-247 (1997).

17. Pilcher, B. K., Sudbeck, B. D., Dumin, J. A., Welgus, H. G., & Parks, W. C. Collagenase-1 and collagen in epidermal repair. *Arch.Dermatol Res.* **290 Suppl**, S37-S46 (1998).

18. Fowlkes, J. L., Enghild, J. J., Suzuki, K., & Nagase, H. Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. *J Biol.Chem.* **269**, 25742-25746 (1994).

19. Fowlkes, J. L., Thrailkill, K. M., Serra, D. M., Suzuki, K., & Nagase, H. Matrix metalloproteinases as insulin-like growth factor binding protein-degrading proteinases. *Prog.Growth Factor Res.* **6**, 255-263 (1995).

20. Noveral, J. P., Bhala, A., Hintz, R. L., Grunstein, M. M., & Cohen, P. Insulin-like growth factor axis in airway smooth muscle cells. *Am J Physiol* **267**, L761-L765 (1994).

21. Jones, J. I. & Clemmons, D. R. Insulin-like growth factors and their binding proteins: biological actions. *Endocr.Rev.* **16**, 3-34 (1995).

22. Gockerman, A., Prevette, T., Jones, J. I., & Clemmons, D. R. Insulin-like growth factor (IGF)-binding proteins inhibit the smooth muscle cell migration responses to IGF-I and IGF-II. *Endocrinology* **136**, 4168-4173 (1995).

23. Rajah, R. *et al.* Elevated levels of the IGF-binding protein protease MMP-1 in asthmatic airway smooth muscle. *Am J Respir Cell Mol.Biol.* **20**, 199-208 (1999).

24. Yamamoto, T., Eckes, B., Mauch, C., Hartmann, K., & Krieg, T. Monocyte chemoattractant protein-1 enhances gene expression and synthesis of matrix metalloproteinase-1 in human fibroblasts by an autocrine IL-1 alpha loop. *J Immunol.* **164**, 6174-6179 (2000).

25. Cheon, H. *et al.* Increased expression of pro-inflammatory cytokines and metalloproteinase-1 by TGF-beta1 in synovial fibroblasts

from rheumatoid arthritis and normal individuals. *Clin.Exp.Immunol.* **127**, 547-552 (2002).

26. Uria, J. A., Jimenez, M. G., Balbin, M., Freije, J. M., & Lopez-Otin, C. Differential effects of transforming growth factor-beta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. *J Biol.Chem.* **273**, 9769-9777 (1998).

27. Ghahary, A. *et al.* Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. *J Invest Dermatol.* **122**, 1188-1197 (2004).

28. Ghahary, A. *et al.* Liposome-associated interferon-alpha-2b functions as an anti-fibrogenic factor for human dermal fibroblasts. *J Invest Dermatol* **109**, 55-60 (1997).

29. Karimi-Busheri, F. *et al.* Expression of a releasable form of annexin II by human keratinocytes. *J Cell Biochem.* **86**, 737-747 (2002).

30. Ghahary, A., Tredget, E. E., Chang, L. J., Scott, P. G., & Shen, Q. Genetically modified dermal keratinocytes express high levels of transforming growth factor-beta1. *J Invest Dermatol* **110**, 800-805 (1998).

31. Moore, B. W. & Perez, V. J. Specific acidic proteins of the nervous system in *Physiological and Biochemical Aspects of Nervous Integration* 343-359. Prentice-Hall, Inc, (2004).

32. Wang, W. & Shakes, D. C. Molecular evolution of the 14-3-3 protein family. *J Mol.Evol.* **43**, 384-398 (1996).

33. Aitken, A., Howell, S., Jones, D., Madrazo, J., & Patel, Y. 14-3-3 alpha and delta are the phosphorylated forms of raf-activating 14-3-3 beta and zeta. In vivo stoichiometric phosphorylation in brain at a Ser-Pro-Glu-Lys MOTIF. *J Biol.Chem.* **270**, 5706-5709 (1995).

34. Aitken, A. *et al.* 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. *Trends Biochem.Sci.* **17**, 498-501 (1992).

35. Fu, H., Subramanian, R. R., & Masters, S. C. 14-3-3 proteins: structure, function, and regulation. *Annu.Rev.Pharmacol.Toxicol.* **40**, 617-647 (2000).

36. Toker, A., Ellis, C. A., Sellers, L. A., & Aitken, A. Protein kinase C inhibitor proteins. Purification from sheep brain and sequence similarity to lipocortins and 14-3-3 protein. *Eur.J Biochem.* **191**, 421-429 (1990).

37. Conklin, D. S., Galaktionov, K., & Beach, D. 14-3-3 proteins associate with cdc25 phosphatases. *Proc.Natl.Acad.Sci.U.S.A* **92**, 7892-7896 (1995).

38. Wakui, H., Wright, A. P., Gustafsson, J., & Zilliacus, J. Interaction of the ligand-activated glucocorticoid receptor with the 14-3-3 eta protein. *J Biol.Chem.* **272**, 8153-8156 (1997).

39. Fantl, W. J. *et al.* Activation of Raf-1 by 14-3-3 proteins. *Nature* **371**, 612-614 (1994).

40. Reuther, G. W., Fu, H., Cripe, L. D., Collier, R. J., & Pendergast, A. M. Association of the protein kinases c-Bcr and Bcr-Abl with proteins of the 14-3-3 family. *Science* **266**, 129-133 (1994).

41. Aitken, A. *et al.* 14-3-3 proteins: a highly conserved, widespread family of eukaryotic proteins. *Trends Biochem.Sci.* **17**, 498-501 (1992).

42. Boston, P. F., Jackson, P., & Thompson, R. J. Human 14-3-3 protein: radioimmunoassay, tissue distribution, and cerebrospinal fluid levels in patients with neurological disorders. *J Neurochem.* **38**, 1475-1482 (1982).

43. Green, A. J. Use of 14-3-3 in the diagnosis of Creutzfeldt-Jakob disease. *Biochem.Soc.Trans.* **30**, 382-386 (2002).

44. Galis, Z. S. & Khatri, J. J. Matrix metalloproteinases in vascular remodeling and atherogenesis: the good, the bad, and the ugly. *Circ.Res.* **90**, 251-262 (2002).

45. Ye, S. Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. *Matrix Biol.* **19**, 623-629 (2000).

46. Feinberg, M. W. *et al.* Transforming growth factor-beta 1 inhibits cytokine-mediated induction of human metalloelastase in macrophages. *J Biol.Chem.* **275**, 25766-25773 (2000).

47. Uria, J. A., Jimenez, M. G., Balbin, M., Freije, J. M., & Lopez-Otin, C. Differential effects of transforming growth factor-beta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. *J Biol.Chem.* **273**, 9769-9777 (1998).

48. Warburton, D. & Bellusci, S. The molecular genetics of lung morphogenesis and injury repair. *Paediatr.Respir.Rev.* **5 Suppl A**, S283-S287 (2004).

49. Holgate, S. T. *et al.* Mechanisms of airway epithelial damage: epithelial-mesenchymal interactions in the pathogenesis of asthma. *Eur.Respir.J Suppl* **44**, 24s-29s (2003).

50. Rosendahl, A. *et al.* Activation of bone morphogenetic protein/Smad signaling in bronchial epithelial cells during airway inflammation. *Am.J Respir.Cell Mol.Biol.* **27**, 160-169 (2002).

51. Zhang, S., Smartt, H., Holgate, S. T., & Roche, W. R. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma. *Lab Invest* **79**, 395-405 (1999).

52. Massagué, J. The transforming growth factor-beta family. *cell biol* **6**, 597-641 (2004).

53. Redington, A. E. *et al.* Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am.J Respir.Crit Care Med.* **156**, 642-647 (1997).

54. Jenkins, H. A. *et al.* Histopathology of severe childhood asthma: a case series. *Chest* **124**, 32-41 (2003).

Chapter 3

Potential regulation of extracellular 14-3-3 proteins by Th2 cytokines

3.1 Th2 Cytokines and Asthma

3.1.1 Introduction

As key cells in the adaptive immune response, T cells coordinate and amplify the effector functions of antigen specific and nonspecific inflammatory cells such as Bcells and eosinophils[1]. T cells have been functionally divided into 2 distinct subsets. The CD4 T cells, termed T-helper cells (Th cells), are involved in humoral immunity, whereas CD8 T cells are referred to as T-suppresser cells and drive the cell-mediated response. The CD4 T cells are particularly important in the regulation of antigen-driven inflammatory processes, although reports have indicated a possible role for the CD8 T cell as well[2].

Many of the inflammatory events associated with atopic disorders such as allergic asthma are orchestrated by Th2 lymphocytes[3], which secrete a variety of cytokines and chemokines that activate eosinophils (IL-3, IL-5, granulocyte macrophage-colony stimulating factor), mast cells (IL-9, IL-4, IL-13), and mucus-producing goblet cells (IL-13) and promote inflammatory cell influx into the airways[4].

Of all TH2 cytokines IL-4 and IL-13 are the most interesting and key cytokines in development of AHR and Atopy in asthma[5]. IL-4 is central to B cells switching to IgE antibody production, and to the maturation of TH cells towards the Th2 phenotype[6]. In addition, IL-13 acts on B cells to produce IgE[7] but also is able to induce the pathophysiological features of asthma in a manner that is independent of immunoglobulin E and eosinophils[8].

3.1.2 IL-4 and IL-13 in Atopy and Asthma

IL4 and IL13 are localized within 25 kb on the proximal portion of chromosome 5q31, to which genome searches have identified strong linkage with asthma and atopy[9,10]. A dinucleotide repeat in the third intron of IL4 is linked to total serum IgE levels but not to asthma in Caucasian populations[11], whereas linkage to both high IgE levels and asthma has been found in a Japanese population[12]. These findings support the candidacy of IL4 as an atopy locus on 5q31.

IL-4 and IL-13 operate through the IL-4 receptor (IL-4R) or IL-13R, respectively[13]. Both receptors share the IL-4Rα chain, and promote STAT-6 (signal transducer and activator of transcription-6) activation[14]. Mice deficient in IL4, IL13, IL4R or Stat6 lack IgE synthesis and Th2-type reactions[15], therefore, genetic variants of IL-4/IL-13 signaling might be crucial for the development of atopic disorders. To date, strong linkages have been identified to flanking markers of the human cytokine gene cluster on 5q31[16,17] and 16p12[18], which include IL4/IL13 and IL4R, respectively. Many groups have tried to identify variants of these genes and to test whether these are associated with asthma or atopy.

Although IL-4 and IL-13 exert similar biological activity[19], IL-13 shows unique activities. Unlike IL-4 deficient mice, IL-13 null mice failed to clear helminths, generate goblet cells responsible for mucus overproduction in asthmatics, or recover basic IgE levels after stimulation with IL-4[20]. Moreover, IL-13 induces

pathophysiological features of asthma independent of IL-4, but dependent on IL-4Rα[21,22]. Thus, neither IL-13 nor IL-4 is redundant and they work in concert to induce atopy.

3.1.3 IL-4 and IL-13 in airway remodeling

Although asthma is an inflammatory disorder of the conducting airways, high-resolution computer tomography and postmortem and biopsy studies have revealed airway wall thickening comprising changes in the epithelium and underlying mesenchyme[23]. These changes which involve epithelial damage, thickening of the lamina reticularis, smooth-muscle hyperplasia, microvascular congestion, edema, and neuronal proliferation are collectively known as airway remodeling[24]. Airway remodeling is likely responsible for profound airway hyperresponsiveness that is sustained in asthma despite prolonged treatment with anti-inflammatory corticosteroids[25-27]. Recent observations in animal models in which airway dysfunction and some aspects of remodeling persist well beyond the resolution of acute inflammatory events[28], suggest that inflammation could disrupt the structural integrity of airways and trigger cascade of remodeling events that continue even in the absence of inflammatory cells.

In this regard, much attention has been focused on contribution of two key T-helper cytokines (IL-4 and IL-13) in development of airway remodeling. In the transgenic model, overexpression of IL-13 in the bronchial epithelium of mice causes goblet-cell metaplasia, subepithelial fibrosis, and smooth-muscle proliferation associated with marked AHR, in addition to lymphocyte and

eosinophil infiltration[29]. Similarly, in a murine model of allergic asthma, blockade of IL-13 using a soluble fusion protein (soluble IL-13α2-IgGFc fusion protein) prevented allergen-induced asthma including increases in mucus cell numbers in the airways[30]. By comparison, mice expressing an IL-4 transgene had goblet-cell metaplasia and high levels of mononuclear cells in the lungs but an absence of airway wall fibrosis or AHR[31].

3.1.4 Contribution of IL-4 and IL-13 to EMTU

Thickening of the lamina reticularis is diagnostic of asthma and reflects events linked to thickening of the entire airway wall[32,33]. In 1990, Holgate et al. described a layer of subepithelial mesenchymal cells with features of myofibroblasts whose number was increased in asthma in proportion to the thickness of the lamina reticularis[34]. More recently the same investigators showed that epithelial epidermal growth factor receptor (EGFR) expression in asthma is positively correlated with the thickness of the lamina reticularis[35], thereby linking epithelial activation and/or injury to the underlying myofibroblast activity. This link is further supported by *in vitro* studies in which injury or mechanical stretching of epithelial monolayers results in increased release of fibroproliferative and fibrogenic growth factors[36,37] whose levels are also increased in asthma[38,39]. Extending these studies into a co-culture model, polyarginine or mechanical damage to confluent monolayers of bronchial epithelial cells grown on a collagen gel seeded with human myofibroblasts leads to enhanced proliferation and increased collagen gene expression from the

combined effects of fibroblast growth factor (FGF)-2, insulin-like growth factor (IGF)-1, platelet derived growth factor (PDGF)-BB, transforming growth factor (TGF)-β, and endothelin (ET)-1[40]. These observations collectively led to the hypothesis that the epithelial mesenchymal trophic unit (EMTU) plays a key role in initiating airway remodelling in asthma as a result of abnormal epithelial injury and repair[41].

The differential effects of IL-4 and IL-13 on airway remodeling and subepithelial fibrosis and the ability of these cytokines to act as profibrogenic factors in asthma have been extensively studied. Data on effects of IL-4 and IL-13 on induction of collagen expression and myofibroblast differentiation is controversial.

In series of *in vitro* studies Saito *et al*[42](2003), reported an increased myofibroblast differentiation in lung fibroblasts treated with either IL-4 or IL-13. This effect was characterized by increased expression of alpha-smooth muscle actin (α-SMA) a myofibroblast marker, in fibroblasts treated with either IL-4 or IL-13[43]. Conversely Richter *et al*[44],2001 in similar set of experiments had shown no significant increase in α-SMA marker in primary bronchial fibroblast culture treated with either IL-4 or IL-13[45]. Moreover the contribution of these TH2 cytokines to airway remodeling was postulated to be due to their functional interaction with EMTU via the epithelium rather than by a direct effect on the submucosal fibroblasts. In the same study both IL-4 and IL-13 significantly stimulate release of TGF-β2 from bronchial epithelial cells[46], suggesting that both cytokines have the potential to use the epithelium to translate remodeling

responses to the underlying mesenchyme. Since expression of IL-4 in the bronchial epithelium in transgenic mice failed to cause subepithelial fibrosis[47], these investigators were not able to explain the fibrogenic effects of IL-4 on fibroblasts via production of TGF-β2 by epithelial cells observed in their *in vitro* model.

3.1.5 Potential regulation of 14-3-3 by IL-4 and IL-13

As discussed earlier chapters, intracellular 14-3-3 proteins have been long known as a group of multifunctional proteins that bind to and modulate a wide array of cellular proteins involved in processes such as metabolism, signal transduction, cell-cycle control, apoptosis, cytoskeletal structure, protein trafficking, transcription, stress response, and malignant transformation[48,49]. Thus far, all the biological activities of 14-3-3 have been identified as intracellular interactions and functions. Recently, Ghahary *et al*(2004) have demonstrated a novel extracellular function for the σ-isotype of 14-3-3[50]. 14-3-3σ isolated form the keratinocytes conditioned medium was shown to stimulate MMP-1 and decrease type I collagen production in dermal fibroblasts. Considering that this keratinocyte-releasable factor has a potent collagenase stimulatory effect on fibroblasts, which favors the resolution of accumulated type I and type III collagen found in fibrotic tissue, these investigators referred to this protein as a keratinocyte-derived anti-fibrogenic factor (KDAF). Subsequently I identified

different releasable isoforms of 14-3-3 proteins (α/β isoform) in lung epithelial cells with similar MMP-1 increasing effect.

Based on antifibrotic properties of KDAF on fibroblasts, we hypothesize that IL-4 and IL-13 could differentially regulate KDAF production in lung epithelial cells. KDAF produced by epithelial cells could work as an anti-fibrogenic factor on the underlying fibroblast layers. The opposite effect of IL-4 and IL-13 on 14-3-3 production maybe able to explain the controversial findings observed between *in vitro* and *in vivo* models of TH2 cytokines induced subepithelial fibrosis.

3.2 Materials and Methods

Cell culture and Reagents:

The human pulmonary type II alveolar epithelial cell line, A549, was purchased from American Type Culture Collection, Rockville, Md and seeded into 75-cm^2 flasks (Corning Inc), maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, New York) supplemented with 10% fetal bovine serum (FBS; GIBCO, Grand Island, NY), 4mmol/L L-glutamine, and 100 μg/ml penicillin/ streptomycin (Gibco) in a humidified 5% CO_2 atmosphere. The medium was replaced 24 hr before experiment with 1% FBS-DMEM, supplemented with 4mmol/L glutamine and 100 μg/ml penicillin/streptomycin. Conditioned medium was collected after 24 hr and subjected to lactate dehydrogenase (LDH) and Western blot analyses. Cells were harvested by brief

treatment with 0.1% trypsin (life technologies Inc., Gaithersburg, MD) and 0.02 % ethylenediaminetetraacetic acid (EDTA) (Sigma, St. Louis, MO) in PBS (PH 7.4) and counted using trypan blue for cell viability.

Lung Fibroblasts: Cells of human diploid fibroblast strain IMR-90, was purchased from American Type Culture Collection (Rockville, MD, USA). Cells were maintained in Eagle Minimum essential medium(EMEM) (Gibco) with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mM non-essential amino acids, and 1.0 mM sodium pyruvate, FBS 10% in a humidified 5% CO_2 atmosphere.

Measurement of LDH in cell supernatants

The concentration of LDH was determined in supernatants of the cultured cells as a marker for cell lysis (Sigma diagnostic kit No.500, colorimetric, endpoint) using a colorimetric enzymatic method. Briefly nicotinamide adenine dinucleotide NAD^+ was reduced to NADH and H^+ by oxidation of lactate to pyruvate. In the second step the catalyst transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT (iodophenyl-nitrophenyl-phenyltetrazolium chloride) which in turn is reduced to formazan. The increase in the amount of enzyme activity in the medium correlates directly with the amount of formazan formed during a limited time period. Thus, the amount of color formed in the assay is proportional to the number of lysed cells. The absorbance of the samples was measured at 450 nm, using a microtiter plate reader. Free medium (DMEM) was used as negative control, and A549 or HS24 cell lysates obtained from each cell culture flask were

used as positive controls in proportion to the amount of condition-medium assayed.

Western blot analysis

Collected conditioned media (CM) were concentrated using a Centricon YM-3 filter device (Millipore Corporation, Beford, MA). After concentration, the volume of the medium was adjusted to cell number using double-distilled water. Concentrated CM was subjected to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) analysis on a 12% (wt/vol) acrylamide gel, and electrotransferred onto PVDF membranes (Millipore Corporation). Active sites on the membranes were blocked in 5% skim milk powder in PBS 0.1% Tween 20 overnight. Immunoblotting was performed using

2μg/ml of goat anti-human 14-3-3 α/β antibody which recognizes an N-terminal peptide of all isoforms of 14-3-3 proteins (Santa Cruz Biotechnology, Inc.). The membranes were then incubated with the appropriate secondary horseradish peroxidase-conjugated anti-goat IgG (Sigma, Saint Louis, MI) (1:2500 dilution). Immunoreactive proteins were then visualized using ECL plus Western blotting detection system (Amersham Biosciences, Buckinghamshire, England).

RNA isolation and Northern blot analysis

Fibroblasts were harvested with 400 μl of 4M guanidium isothiocynate (GITC) solution and total RNA from each group was isolated by the acid-guanidium-phenol chloroform method [51]. Total RNA from each individual fibroblast culture

was then separated by electrophoresis (10 μg per lane) on a 1% agarose gel containing 2.2 M formaldehyde and was blotted onto a nitrocellulose membrane (Bio- Rad Laboratories, Hercules, California). The blots were baked for 2hr at 80°C under vacuum and prehybridized for 4hr at 45°C in a prehybridization solution. Hybridization was performed at 45°C in the same solution, using collagenase cDNA probes. The cDNA probe for collagenase was obtained from the American Type Culture Collection (Rockville, MD) and labelled with P-α^{32}-dCTP by nick translation. The filters were washed initially at room temperature with 2X sodium citrate/sodium chloride buffers and 0.1% sodium dodecylsulfate for 1hr and finally washed for 20 min at 65°C in 0.1X sodium citrate/sodium chloride buffer and 0.1% sodium dodecylsulfate. Autoradiography was performed by exposing Kodak X-Omat film to nitrocellulose filters at -80°C in the presence of intensifying screen. Quantitative analysis of autoradiographs was accomplished by densitometry.

3.3 Results

3.3.1 IL-4 increases 14-3-3α/β production at the mRNA and the protein level

To study the effect of IL-4 on 14-3-3α/β production, A549 epithelial cells were grown in six well plates and treated with DMEM + 10% FBS until confluent. The media was then changed to DMEM + 1% FBS and cells were then incubated for 24 hr with or without IL-4 treatment (20 ng/ml). Conditioned media (A549 CM) was collected and subjected to Western blot analysis for detection of 14-3-3α/β . As shown in figure 3.1, A, treatment of A549 epithelial cells with IL-4 resulted in a 4-fold increase of 14-3-3 production (densitometry data not shown), at the protein level. Further A549 CM, was assayed for the presence of lactate dehydrogenase (LDH) as a marker of cell lysis[52,53]. As shown in figure 3.1,B, A549 CM (+IL-4) contained 0.25% of the total LDH released from epithelial cell lysate within each plate, A549 CM (-IL-4) contained 0.3% of the total LDH respectively. I therefore established that the release of 14-3-3 proteins into the media was not the result of epithelial cell lysis. In separate set of experiments A549 epithelial cells were incubated in 1% FBS containing DMEM upon confluence and incubated with or without IL-4 (20 ng/ml) for 24hr. Total RNA was then extracted form epithelial cells and expression of 14-3-3α/β mRNA was evaluated by Northern analysis. As shown in figure 3.2,A, there is a 2 fold increase in expression of 14-3-3α/β at the mRNA level. Figure 3.2, C, shows the densitometry results of 14-3-3mRNA.

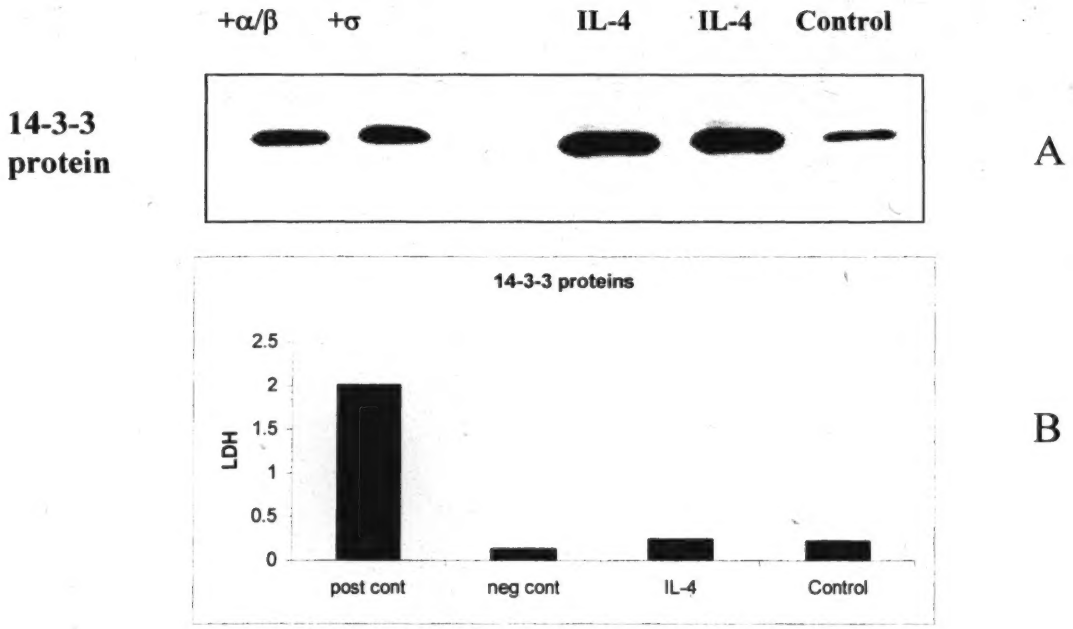


Figure 3.1: A, Western blot analysis of A549 CM, with or with out treatment with IL-4 (24 hrs) for detection of all isoforms of 14-3-3 (n = 1). B: LDH assay performed on CM of A549 following incubation in low FBS medium for 24hrs,Post cont: Total A549 cell lysis, Negative control: free media, IL-4: conditioned media from A549 cells treated with IL-4, Control: Conditioned media of A549 cells with out IL-4 treatment

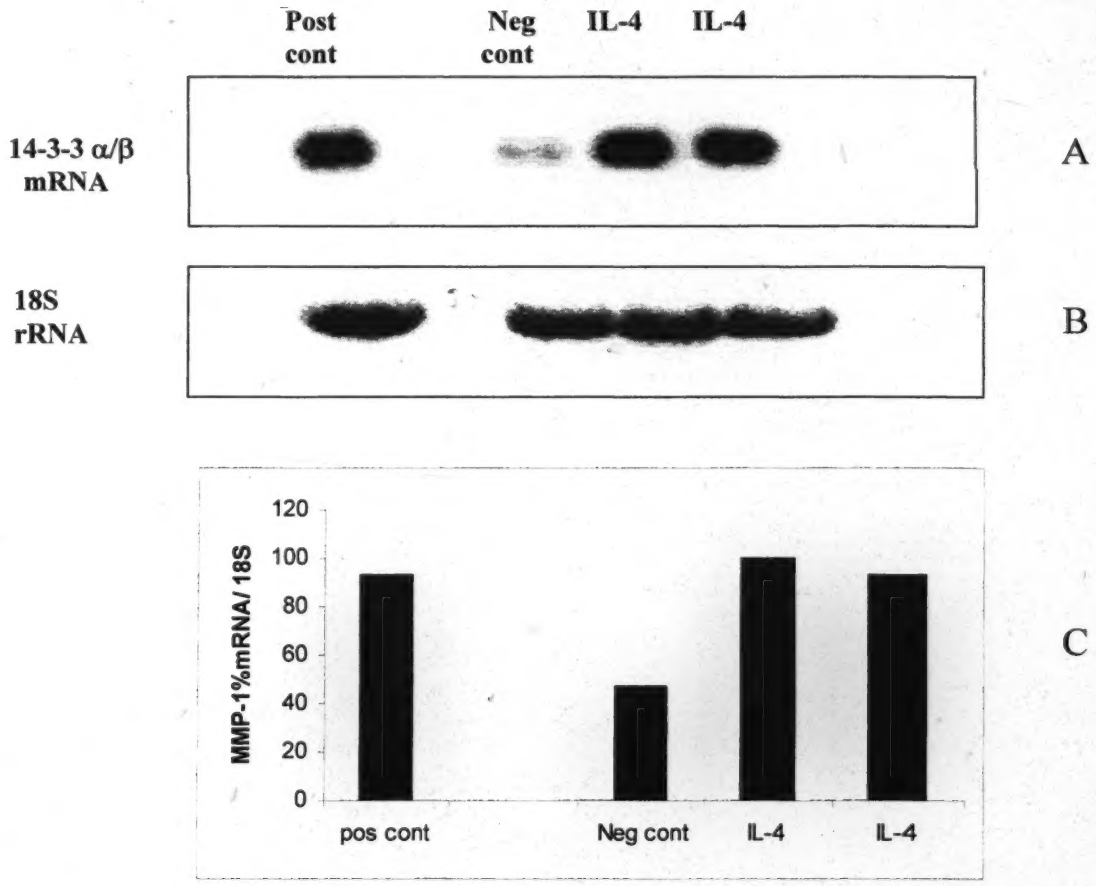


Figure 3.2; A: Northernblot analysis for detection of 14-3-3 mRNA expression in A549 epithelial cells treated with or without IL-4 (20ng/ml). B: Loading control with 18S ribosomal RNA. C. Densitometry analysis of 14-3-3 bands. Lane 1: Positive recombinant 14-3-3α/β, , Lane2: No treatment, Lane 3&4: IL-4 treatment

3.3.2 IL-13 decrease 14-3-3 α/β production at the protein level

To compare the effect of IL-13 with that of IL-4 on regulation of KDAF production from lung epithelial cells, a series of similar studies was conducted using IL-13 treatment. Briefly A549 epithelial cells were grown in six well plates and treated with DMEM + 10% FBS until confluent. The media was then changed to DMEM + 1% FBS and cells were then incubated for 24 hr with or without IL-13 treatment (20 ng/ml). Conditioned media (A549 CM) was collected and subjected to Western blot analysis for detection of 14-3-3σ. As shown in figure 3.3,A, and in contrast to IL-4 treatment study, expression of 14-3-3σ was markedly decreased upon treatment with IL-13 for 24hr.Figure 3.3,B shows the result of Western analysis on two separate experiments.

Similar to the previous experiments A549 CM was assayed for the presence of LDH. As shown in Figure 3.3, C, A549 CM (+IL-13) contained 0.3% of the total LDH released and A549 CM (-IL-13) contained 0.28% respectively, confirming that the 14-3-3 detected in the CM is not due to epithelial cell lysis.

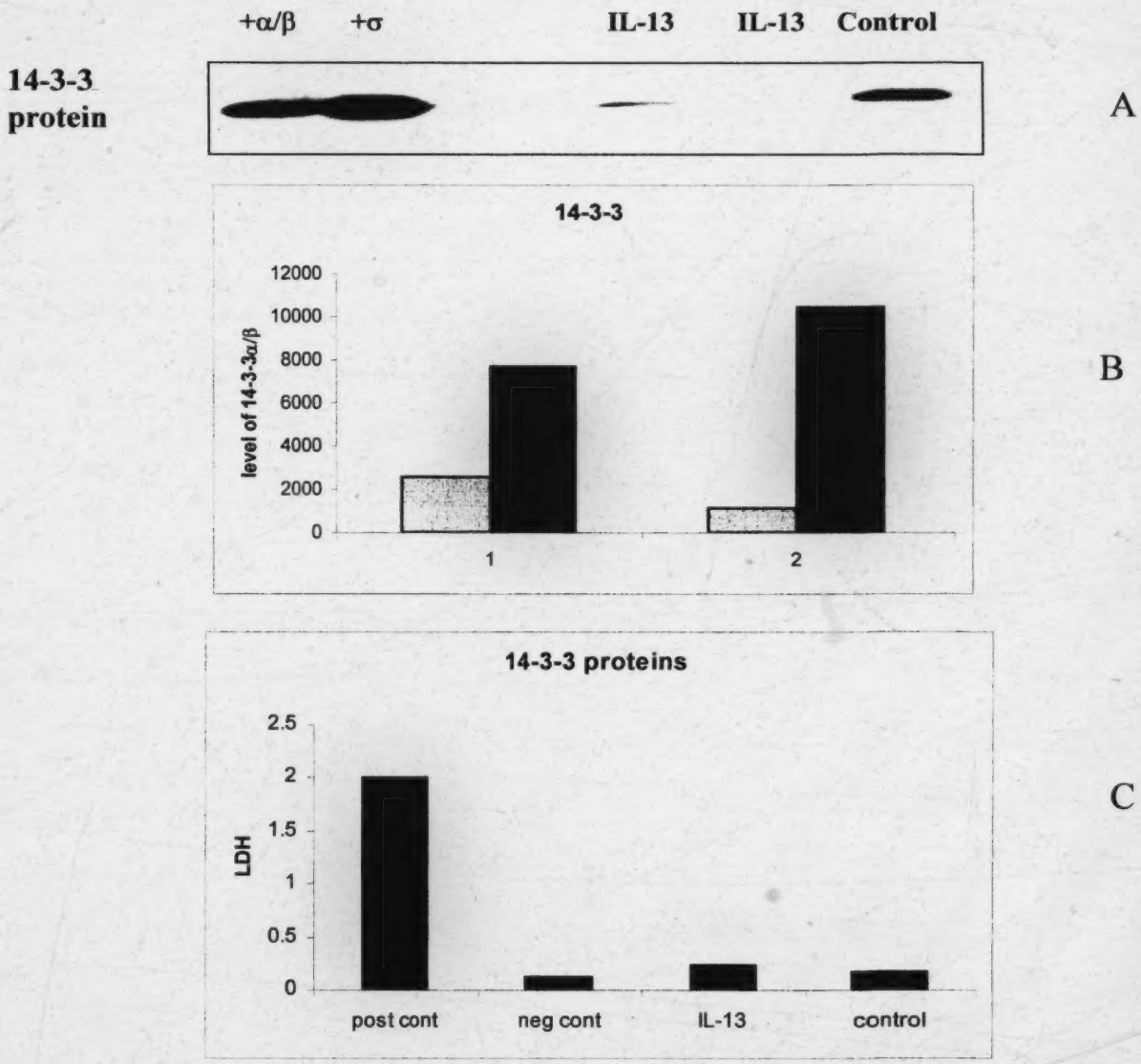


Figure 3.3: A, Western blot analysis of A549 CM, with or with out treatment with IL-13 (20 ng/ml) (24 hrs) for detection of all isoforms of 14-3-3 (n = 2). B: Densitometry results on Western analysis of 2 different experiments, Hatched: A549 treated with IL-13, Filled: A549 not treated. C: LDH assay performed on CM of A549 following incubation in low FBS medium for 24hrs, Positive control (post cont): Total A549 cell lysis, Negative control: free media, IL-13: conditioned media from A549 cells treated with IL-13, Control: Conditioned media of A549 cells with out IL-13 treatment

3.4 Discussion

Asthma is a complex genetic disease with multiple genes interacting with the environment to modify both susceptibility and severity of disease. IL-4 and IL-13 appear to play key roles in this genetic susceptibility with promoter and/or functional polymorphisms in their own genes, those of their composite receptors, and their intracellular mediator, signal transducer and activator of transcription-6, many of which have been linked to asthma[54]. Whereas the effects of IL-4 and IL-13 on T- and B-cell switching can explain their pro-allergic effects in asthma, observations derived from experimental animals suggest that IL-13 may play a more important role than IL-4 in airway remodeling through its effects on subepithelial fibrosis and bronchial hyperresponsiveness (BHR). Recent studies have shown that BHR characterized by sustained airway dysfunction is a consequence of airway remodeling rather than ongoing cellular inflammation[55]. An increase in fibrosis just below the large airway epithelium occurs as a prominent feature in asthma. This layer has been extensively studied due to its accessibility on endobronchial biopsy. The normal layer of collagen under the airway is ~5 μm thick, which increases to 7–23 μm in patients with asthma. Initially described as basement membrane thickening, it is now apparent that the true basement membrane (the lamina rara and densa as seen on electron microscopy, which contain laminin and collagen IV) is not grossly altered[56]. There is, rather, thickening of the area just below the true basement membrane, the lamina reticularis, with deposition of interstitial collagens I, III, and V[57].

The significance of this specific feature of asthmatic airway remodeling is unclear. Although measurements of airway distensibility correlate well with subepithelial fibrosis[58], other functional measurements (clinical illness scores, measures of pulmonary function and airway hyperresponsiveness) show variable correlations[59-61]. Many investigators have identified both severe asthmatics with no increase in subepithelial fibrosis and nonasthmatics with increased subepithelial fibrosis[62,63]. Subepithelial fibrosis is actually a very early marker for the asthmatic phenotype in children and does not correlate with length of time with the disease nor necessarily with the severity of inflammation[64-66]. It has therefore been suggested that subepithelial fibrosis represents disordered epithelial-mesenchymal signaling rather than a direct response to inflammatory injury[67]. A tracheal explant model has shown that cigarette smoke can induce remodeling in the absence of inflammation, indicating that other pathways to fibrosis need to be considered[68].

Our group has previously shown that extracellular form of 14-3-3 proteins (α/β and σ isoforms) is an important factor in epithelial-mesenchymal communication[50]. 14-3-3σ released form dermal epithelial cells (keratinocytes) can significantly increase the production of MMP-1 from dermal fibroblasts. We subsequently showed that in lung, epithelial cells released 14-3-3β but not the σ isoform and significantly induce MMP-1 expression in lung fibroblasts in a dose dependent manner (chapter 2). As discussed in previous chapters, in lung MMP-1 is mainly secreted by fibroblasts and activated monocytes and macrophages[69]. MMP-1 is an important collagenase which levels are increased in asthma and has

been correlated with the disease severity[70]. Regulation of MMPs expression and production is the result of a complex network of reciprocal interactions between different mediators and cells. Among these pathways, monocyte chemoattractant protein-1 (MCP-1) and transforming growth factor-β_1 (TGF-β_1), two mediators reported to be increased in the bronchial tree from asthmatics[71,72] have been well studied. However these pathways are controlled via inflammatory mediators and therefore could not be solely responsible for airway remodeling observed in the absence of inflammation[73]. Discovery of extracellular 14-3-3 proteins as an epithelial cell derived factor which could modulate MMP-1 expression in lung fibroblasts, brings about many interesting questions. It was important to determine if TH_2 inflammatory cytokines such as IL-4 and IL-13 have any effect on releasable 14-3-3 from lung epithelial cells, thereby altering the EMTU communication which could potentially result in airway remodeling even long after the primary inflammatory process has been subsided.

In current study, my preliminary results show that IL-4 and IL-13 do change the releasable 14-3-3 protein profile in the conditioned medium of A549 lung epithelial cell lines. I have shown that where treatment of epithelial cells with IL-4 results in increased measurement of extracellular 14-3-3 at the protein level, similar treatment with IL-13 significantly reduces 14-3-3 proteins in epithelia cell-conditioned medium. Previous *in vitro* studies with IL-4 and IL-13 have described both cytokines as profibrogenic factors, due to their ability to release TGF-β_2 from primary bronchial epithelial cells in a corticosteroid-insensitive manner[74]. However *in vivo* models did not support a consistent effect between

IL-4 and IL-13 in induction of airway remodeling. Although overexpression of IL-13 in the bronchial epithelium of mice causes goblet-cell metaplasia, subepithelial fibrosis, and smooth-muscle proliferation associated with marked BHR [75], and blockade of IL-13 using a soluble fusion protein in a murine model of allergic asthma prevents allergen-induced asthma including increase in mucus cell numbers in the airways[76], different effects are observed in IL-4 transgenic model. Mice expressing an IL-4 transgene had goblet-cell metaplasia and high levels of mononuclear cells in the lungs but an absence of airway wall fibrosis or BHR[77]. These results imply that with regards to effect of TH_2 cytokines on airway remodeling, IL-4 and IL-13 may alter different anti or pro fibrogenic pathways other than TGF-β pathway which are still not well understood.

Here, I have shown for the first time that IL-4 and IL-13 can modulate epithelial cell function through release of 14-3-3 proteins. However, the results of this study remain in need of validation, as I was unable to show the IL-4 and IL-13 effects reproducibly in other experiments. Lung epithelial cell treatment with IL-4 increased 14-3-3 release in conditioned medium. We can therefore postulate that the absence of subepithelial fibrosis in transgenic IL-4 mice could be the result of excessive release of 14-3-3 protein from airway epithelial layer, resulting in increased production of MMP-1 which in turn could degrade the deposited subepithelial fibrillar collagen. In contrast IL-13 treatment significantly decreased epithelial cell-released 14-3-3 proteins, which can result in reduction of MMP-1 production by underlying fibroblast layer and decrease the rate of collagen degradation.

In conclusion, IL-4 and IL-13 are key cytokines that contribute to asthma severity and chronicity by augmenting responses within the EMTU. The availability of *in vitro* systems of human asthmatic bronchial epithelial cells and fibroblasts will enable detailed characterization of these interactions as well as early evaluation of novel, targeted interventions directed toward the aberrant responses of airway structural cells.

3.5 Reference List

1. Eisenbarth, S. C., Cassel, S., & Bottomly, K. Understanding asthma pathogenesis: linking innate and adaptive immunity. *Curr.Opin.Pediatr.* **16**, 659-666 (2004).

2. O'Brien, R. M., Xu, H., Rolland, J. M., Byron, K. A., & Thomas, W. R. Allergen-specific production of interferon-gamma by peripheral blood mononuclear cells and CD8 T cells in allergic disease and following immunotherapy. *Clin.Exp.Allergy* **30**, 333-340 (2000).

3. Busse, W. W. & Lemanske, R. F., Jr. Asthma. *N.Engl.J Med* **344**, 350-362 (2001).

4. Strachan, D. P. Hay fever, hygiene, and household size. *BMJ* **299**, 1259-1260 (1989).

5. Chomarat, P. & Banchereau, J. Interleukin-4 and interleukin-13: their similarities and discrepancies. *Int.Rev.Immunol.* **17**, 1-52 (1998).

6. Izuhara, K. & Shirakawa, T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. *Int.J Mol.Med* **3**, 3-10 (1999).

7. Izuhara, K. & Shirakawa, T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. *Int.J Mol.Med* **3**, 3-10 (1999).

8. Wills-Karp, M. *et al.* Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258-2261 (1998).

9. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA). *Nat.Genet.* **15**, 389-392 (1997).

10. Ober, C. *et al.* Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. *Hum.Mol.Genet.* **7**, 1393-1398 (1998).

11. Marsh, D. G. *et al.* Linkage analysis of IL4 and other chromosome 5q31.1 markers and total serum immunoglobulin E concentrations. *Science* **264**, 1152-1156 (1994).

12. Noguchi, E. *et al.* Evidence for linkage between asthma/atopy in childhood and chromosome 5q31-q33 in a Japanese population. *Am J Respir Crit Care Med* **156**, 1390-1393 (1997).

13. Izuhara, K. & Shirakawa, T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. *Int.J Mol.Med* **3**, 3-10 (1999).

14. Izuhara, K. & Shirakawa, T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. *Int.J Mol.Med* **3**, 3-10 (1999).

15. Izuhara, K. & Shirakawa, T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. *Int.J Mol.Med* **3**, 3-10 (1999).

16. A genome-wide search for asthma susceptibility loci in ethnically diverse populations. The Collaborative Study on the Genetics of Asthma (CSGA). *Nat.Genet.* **15**, 389-392 (1997).

17. Ober, C. *et al.* Genome-wide search for asthma susceptibility loci in a founder population. The Collaborative Study on the Genetics of Asthma. *Hum.Mol.Genet.* **7**, 1393-1398 (1998).

18. Deichmann, K. A. *et al.* Linkage and allelic association of atopy and markers flanking the IL4-receptor gene. *Clin.Exp.Allergy* **28**, 151-155 (1998).

19. Izuhara, K. & Shirakawa, T. Signal transduction via the interleukin-4 receptor and its correlation with atopy. *Int.J Mol.Med* **3**, 3-10 (1999).

20. McKenzie, G. J., Bancroft, A., Grencis, R. K., & McKenzie, A. N. A distinct role for interleukin-13 in Th2-cell-mediated immune responses. *Curr.Biol.* **8**, 339-342 (1998).

21. Grunig, G. *et al.* Requirement for IL-13 independently of IL-4 in experimental asthma. *Science* **282**, 2261-2263 (1998).

22. Wills-Karp, M. *et al.* Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258-2261 (1998).

23. Mitsunobu, F. & Tanizaki, Y. The use of computed tomography to assess asthma severity. *Curr.Opin.Allergy Clin.Immunol.* **5**, 85-90 (2005).

24. James, A. Airway remodeling in asthma. *Curr.Opin.Pulm.Med* **11**, 1-6 (2005).

25. Sont, J. K. *et al.* Clinical control and histopathologic outcome of asthma when using airway hyperresponsiveness as an additional guide

to long-term treatment. The AMPUL Study Group. *Am J Respir Crit Care Med* **159**, 1043-1051 (1999).

26. Jeffery, P. K. *et al.* Effects of treatment on airway inflammation and thickening of basement membrane reticular collagen in asthma. A quantitative light and electron microscopic study. *Am Rev.Respir Dis.* **145**, 890-899 (1992).

27. Djukanovic, R. *et al.* Effect of an inhaled corticosteroid on airway inflammation and symptoms in asthma. *Am Rev.Respir Dis.* **145**, 669-674 (1992).

28. Leigh, R. *et al.* Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol.Biol.* **27**, 526-535 (2002).

29. Zhu, Z. *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin.Invest* **103**, 779-788 (1999).

30. Wills-Karp, M. *et al.* Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258-2261 (1998).

31. Rankin, J. A. *et al.* Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc.Natl.Acad.Sci U.S.A* **93**, 7821-7825 (1996).

32. James, A. L., Maxwell, P. S., Pearce-Pinto, G., Elliot, J. G., & Carroll, N. G. The relationship of reticular basement membrane thickness to airway wall remodeling in asthma. *Am J Respir Crit Care Med* **166**, 1590-1595 (2002).

33. Ward, C. *et al.* Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma. *Am J Respir Crit Care Med* **164**, 1718-1721 (2001).

34. Brewster, C. E. *et al.* Myofibroblasts and subepithelial fibrosis in bronchial asthma. *Am J Respir Cell Mol.Biol.* **3**, 507-511 (1990).

35. Puddicombe, S. M. *et al.* Involvement of the epidermal growth factor receptor in epithelial repair in asthma. *FASEB J* **14**, 1362-1374 (2000).

36. Tschumperlin, D. J., Shively, J. D., Kikuchi, T., & Drazen, J. M. Mechanical stress triggers selective release of fibrotic mediators from bronchial epithelium. *Am J Respir Cell Mol.Biol.* **28**, 142-149 (2003).

37. Zhang, S., Smartt, H., Holgate, S. T., & Roche, W. R. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma. *Lab Invest* **79**, 395-405 (1999).

38. Redington, A. E. *et al.* Basic fibroblast growth factor in asthma: measurement in bronchoalveolar lavage fluid basally and following allergen challenge. *J Allergy Clin.Immunol.* **107**, 384-387 (2001).

39. Redington, A. E. *et al.* Transforming growth factor-beta 1 in asthma. Measurement in bronchoalveolar lavage fluid. *Am J Respir Crit Care Med* **156**, 642-647 (1997).

40. Zhang, S., Smartt, H., Holgate, S. T., & Roche, W. R. Growth factors secreted by bronchial epithelial cells control myofibroblast proliferation: an in vitro co-culture model of airway remodeling in asthma. *Lab Invest* **79**, 395-405 (1999).

41. Holgate, S. T. *et al.* Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin.Immunol.* **105**, 193-204 (2000).

42. Saito, A., Okazaki, H., Sugawara, I., Yamamoto, K., & Takizawa, H. Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. *Int.Arch Allergy Immunol.* **132**, 168-176 (2003).

43. Saito, A., Okazaki, H., Sugawara, I., Yamamoto, K., & Takizawa, H. Potential action of IL-4 and IL-13 as fibrogenic factors on lung fibroblasts in vitro. *Int.Arch Allergy Immunol.* **132**, 168-176 (2003).

44. Richter, A. *et al.* The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol.Biol.* **25**, 385-391 (2001).

45. Richter, A. *et al.* The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol.Biol.* **25**, 385-391 (2001).

46. Richter, A. *et al.* The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol.Biol.* **25**, 385-391 (2001).

47. Rankin, J. A. *et al.* Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc.Natl.Acad.Sci U.S.A* **93**, 7821-7825 (1996).

48. Xiao, B. *et al.* Structure of a 14-3-3 protein and implications for coordination of multiple signalling pathways. *Nature* **376**, 188-191 (1995).

49. Muslin, A. J. & Xing, H. 14-3-3 proteins: regulation of subcellular localization by molecular interference. *Cell Signal.* **12**, 703-709 (2000).

50. Ghahary, A. *et al.* Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. *J Invest Dermatol.* **122**, 1188-1197 (2004).

51. Ghahary, A., Tredget, E. E., Chang, L. J., Scott, P. G., & Shen, Q. Genetically modified dermal keratinocytes express high levels of transforming growth factor-beta1. *J Invest Dermatol* **110**, 800-805 (1998).

52. Korzeniewski, C. & Callewaert, D. M. An enzyme-release assay for natural cytotoxicity. *J Immunol.Methods* **64**, 313-320 (1983).

53. Decker, T. & Lohmann-Matthes, M. L. A quick and simple method for the quantitation of lactate dehydrogenase release in measurements of cellular cytotoxicity and tumor necrosis factor (TNF) activity. *J Immunol.Methods* **115**, 61-69 (1988).

54. Shirakawa, I. *et al.* Atopy and asthma: genetic variants of IL-4 and IL-13 signalling. *Immunol.Today* **21**, 60-64 (2000).

55. Leigh, R. *et al.* Dysfunction and remodeling of the mouse airway persist after resolution of acute allergen-induced airway inflammation. *Am J Respir Cell Mol.Biol.* **27**, 526-535 (2002).

56. Roche, W. R., Beasley, R., Williams, J. H., & Holgate, S. T. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* **1**, 520-524 (1989).

57. Roche, W. R., Beasley, R., Williams, J. H., & Holgate, S. T. Subepithelial fibrosis in the bronchi of asthmatics. *Lancet* **1**, 520-524 (1989).

58. Ward, C. *et al.* Reduced airway distensibility, fixed airflow limitation, and airway wall remodeling in asthma. *Am J Respir Crit Care Med* **164**, 1718-1721 (2001).

59. Hoshino, M., Nakamura, Y., Sim, J., Shimojo, J., & Isogai, S. Bronchial subepithelial fibrosis and expression of matrix metalloproteinase-9 in asthmatic airway inflammation. *J Allergy Clin.Immunol.* **102**, 783-788 (1998).

60. Cho, S. H. *et al.* Pathological changes according to the severity of asthma. *Clin.Exp.Allergy* **26**, 1210-1219 (1996).

61. Benayoun, L., Druilhe, A., Dombret, M. C., Aubier, M., & Pretolani, M. Airway structural alterations selectively associated with severe asthma. *Am J Respir Crit Care Med* **167**, 1360-1368 (2003).

62. Wenzel, S. E. *et al.* Evidence that severe asthma can be divided pathologically into two inflammatory subtypes with distinct physiologic and clinical characteristics. *Am J Respir Crit Care Med* **160**, 1001-1008 (1999).

63. Chakir, J. *et al.* Lower airways remodeling in nonasthmatic subjects with allergic rhinitis. *Lab Invest* **75**, 735-744 (1996).

64. Payne, D. N. *et al.* Early thickening of the reticular basement membrane in children with difficult asthma. *Am J Respir Crit Care Med* **167**, 78-82 (2003).

65. Cokugras, H. *et al.* Ultrastructural examination of bronchial biopsy specimens from children with moderate asthma. *Thorax* **56**, 25-29 (2001).

66. Boulet, L. P. *et al.* Airway hyperresponsiveness, inflammation, and subepithelial collagen deposition in recently diagnosed versus long-standing mild asthma. Influence of inhaled corticosteroids. *Am J Respir Crit Care Med* **162**, 1308-1313 (2000).

67. Holgate, S. T. *et al.* Epithelial-mesenchymal interactions in the pathogenesis of asthma. *J Allergy Clin.Immunol.* **105**, 193-204 (2000).

68. Wang, R. D. *et al.* Cigarette smoke produces airway wall remodeling in rat tracheal explants. *Am J Respir Crit Care Med* **168**, 1232-1236 (2003).

69. Emonard, H. *et al.* Interactions between fibroblasts and a reconstituted basement membrane matrix. *J Invest Dermatol.* **89**, 156-163 (1987).

70. Cataldo, D. D. *et al.* Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases mRNA transcripts in the bronchial secretions of asthmatics. *Lab Invest* **84**, 418-424 (2004).

71. Sousa, A. R. *et al.* Increased expression of the monocyte chemoattractant protein-1 in bronchial tissue from asthmatic subjects. *Am J Respir Cell Mol.Biol.* **10**, 142-147 (1994).

72. Vignola, A. M. *et al.* Transforming growth factor-beta expression in mucosal biopsies in asthma and chronic bronchitis. *Am J Respir Crit Care Med* **156**, 591-599 (1997).

73. Jenkins, H. A. *et al.* Histopathology of severe childhood asthma: a case series. *Chest* **124**, 32-41 (2003).

74. Richter, A. *et al.* The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol.Biol.* **25**, 385-391 (2001).

75. Zhu, Z. *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin.Invest* **103**, 779-788 (1999).

76. Wills-Karp, M. *et al.* Interleukin-13: central mediator of allergic asthma. *Science* **282**, 2258-2261 (1998).

77. Rankin, J. A. *et al.* Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc.Natl.Acad.Sci U.S.A* **93**, 7821-7825 (1996).

Chapter 4

General discussion and conclusion

4.1 Discussion and Conclusion

The ability to generate or repair injured tissue is essential to the continuity of human life. In the lung, epidermal-mesenchymal interactions play a critical role in modulating the expression of MMPs during both development and bronchial healing process. Disruption of this interaction, as in case of asthma, may cause alterations in bronchial structures that are collectively referred to as airway remodeling.

In a previous study to identify MMP-1 stimulating factors produced by epithelial cells in skin (keratinocytes), Ghahary *et al* (2004) isolated a keratinocyte-derived factor from keratinocyte-conditioned medium and subsequently identified it as stratifin (14-3-3σ), which has potent MMP-1 stimulatory effects on dermal fibroblasts[1]. Further, these investigators show that 14-3-3σ-induced MMP-1 enhancement in fibroblasts are mediated through the p38 mitogen-activated protein kinase (MAPK) pathway (E.Lam *et al*, 2005, Journal of Investigative Dermatology, in press). With regard to extracellular stratifin regulation, the same investigators showed that insulin is able to suppress the MMP-1 stimulatory effect of extracellular 14-3-3σ[2]. In lung MMP-1 is an important enzyme that degrades the interstitial types I, II, and III collagens, and is also involved in the cleavage of a number of non-matrix substrates and cell surface molecules[3]. In normal adult tissues, the levels of MMP-1 are usually low. By contrast, MMP-1 expression is elevated when the system faces a disturbance, such as wound healing, repair, or remodeling processes as occur in several pathological conditions including asthma[4-8]. Expression of MMP-1 is regulated at different levels. It is transcriptionally up-regulated by a variety of different growth factors, hormones, and cytokines including epidermal growth factor, fibroblast growth factor (FGF)-1, -2, -7, and -9, hepatocyte growth factor,

granulocyte-macrophage colony-stimulating factor, interferon beta and gamma, platelet-derived growth factor, transforming growth factor (TGF) alpha, monocyte chemoattractant protein-1 (MCP-1), and interleukins (IL)-1, -4, -5, -6, -8, and -10[4,9]. On the other hand, TGFβ, a proinflammatory cytokine, shown to be increased in asthmatic airways, the vitamin A-derived all-trans retinoic acid, and the synthetic retinoids all suppress MMP-1 transcription[10].

Previously known as multifunctional intracellular factors, 14-3-3 proteins are involved in protein trafficking, signal transduction and other subcellular functions. Discovery of extracellular 14-3-3σ, a MMP-1 inducer in dermal fibroblasts, opens yet another interesting avenue in MMP-1 regulatory mechanisms. However, no information concerning the extracellular function of 14-3-3σ or other isoforms of 14-3-3 family of proteins in lung has been determined. Moreover, no information is available about the net effect of these proteins on lung fibroblasts in the presence of MMP-1 inhibitory factors such as TGF-β_1. Further, the potential effect of Th2 inflammatory cytokines on regulation of epithelial cell-derived 14-3-3 proteins was not studied. Therefore, this thesis represents an attempt to identify and examine the extracellular effects of lung epithelial cell derived-14-3-3 proteins on lung fibroblast MMP-1 expression alone, or in the presence of TGF-β_1, a known fibrogenic factor which decreases MMP-1 expression in lung fibroblasts. Further, I studied the regulation of 14-3-3 protein release in lung epithelial cells by IL-4 and IL-13 cytokines, *in vitro*.

In regard to investigating the presence of 14-3-3 proteins in lung epithelial cell conditioned medium (LCM), I first observed that despite the abundant presence of 14-3-3 proteins, and in contrast to that of keratinocytes, the σ isoform of 14-3-3 proteins is not

present in LCM. Further, I showed that similar to stratifin, LCM is able to significantly induce MMP-1 production in dermal fibroblasts, despite the absence of 14-3-3σ. I subsequently repeated the experiment using lung fibroblasts. My results show that treatment with LCM significantly upregulated MMP-1 mRNA expression in lung fibroblasts (IMR-90).

To determine the importance of extracellular 14-3-3 in the induction of MMP-1, we immunodepleted LCM of 14-3-3 proteins (using an antibody that recognize all isoforms), and showed a significant decrease in expression of MMP-1 mRNA following treatment with 14-3-3 depleted LCM. This result confirms my hypothesis that extracellular 14-3-3 proteins are the most potent collagenase inducing factor in lung conditioned medium.

However, unlike findings of Ghahary *et al* (2004), with regard to MMP-1, lung fibroblasts did not respond to 14-3-3σ treatment. Interestingly, I showed that a different isoform of 14-3-3 proteins (α/β) could recapitulate the MMP-1-inducing effect of stratifin(14-3-3σ) in lung. This finding corresponds well with our early observation that 14-3-3σ is absent in LCM. I therefore postulate that upregulation of MMP-1 expression in different organs are isoform dependent.

This observation has an important clinical implication. MMP-1 is one of only four members of MMP family (MMP-1, MMP-8, MMP-13 and MMP-14 (MT1-MMP)) that can degrade fibrillar collagens in their triple-helical domain, leaving molecules unstable and ready for further degradation by other MMPs[3]. Additionally, other matrix molecules, including aggrecan, versican, perlecan, casein, nidogen, serpins, and tenascin-C[11] are substrates for MMP-1. Therefore, MMP-1 should play a pivotal role in the physiologic and pathologic remodeling of extracellular matrix. Moreover in the recent years it has

become clear that beside extracellular matrix proteins, MMPs are also able to cleave non-matrix substrates form their associated binding proteins[12-15]. In this regard cleavage of the proteoglycan perlecan by MMP-1 can release fibroblast growth factor (FGF)[15] an important factor in remodeling and fibroblast activation in airways. Likewise, cleavage of insulin-like growth factor (IGF) from their binding protein (IGF-BP), result in release of active IGF ligands. IGF proteins are major regulator of airway smooth muscle (ASM) cells growth and proliferation[16], activation of these ligands will ultimately lead to airway smooth muscle hyperplasia , an important histopathologic feature of chronic asthma. Taken together, understanding the mechanisms behind the MMP-1 upregulation in different organs of the body, and discovering potential inhibitors of these pathways are essential for further therapeutic interventions to prevent airway tissue remodeling.

As mentioned above, immunodepletion of 14-3-3 proteins from epithelial conditioned medium, the milieu which fibroblasts are constantly exposed to, results in a significant decrease of MMP-1 expression at the transcriptional level. Inhibition of 14-3-3 release from epithelial cells may be a key to decrease MMP-1 expression and therefore be a potential candidate to prevent airway tissue remodeling.

To further assess 14-3-3 potency, I studied the stimulatory effect of 14-3-3 α/β on MMP-1 expression in lung fibroblasts, in the presence of TGF-β_1. My results show that the inhibitory role of TGF-β_1 on MMP-1 expression did not appear to decrease the 14-3-3α/β effect markedly, suggesting a potent collagenase-stimulatory role for the extracellular form of 14-3-3α/β that may override the inhibitory effect of TGF-β_1. TGF-β_1 is temporally associated with myofibroblast formation and the expression of tenascin and

procollagen-I by fibroblasts in allergic inflammation[17]. With regard to collagenase, TGF-β_1 has been shown to overall decrease MMP-1 production of fibroblasts[18].

The persistence of high MMP-1 mRNA in fibroblasts treated with 14-3-3α/β and TGF-β_1, shows that with respect to collagenase, 14-3-3 α/β overpowers the effect of TGF-β_1.

In addition, my results showing up-regulation of 14-3-3α/β by IL-4, and its down regulation by IL-13, may be a step in explaining the observations made in transgenic animal models. IL-4 and IL-13 exert similar biological activities[19] and they are both considered profibrogenic factors due to their ability to release TGF-β_2 from bronchial epithelial cells *in vitro*[20]. However, evidence of subepithelial fibrosis is only evident in IL-13 transgenic mice[21] and not in the IL-4 model[22]. Given the potent effect of 14-3-3α/β on MMP-1 expression, I concluded that high levels of this protein in IL-4 transgenic mice may result in over expression of MMP-1 and could possibly be the reason for lack of subepithelial fibrosis in these animals.

Since the discovery of 14-3-3 proteins many intracellular activities have been attributed to this multifunctional family of proteins[23]. The extracellular presence of these proteins such as in cerebrospinal fluid (CSF) has been known for some time[24]. However until recently no biological activities have been attributed to extracellular form of 14-3-3α/β protein. My results describe for the first time, a novel MMP-1 inducing factor involved in epithelial-mesenchymal communication in the lung.

The precise clinical significance of airway remodeling is currently debated and the complex mechanism behind airway structural changes in the course of chronic is not well understood. The asthma paradigm of "inflammation causing remodeling" has taken new turns by recent discoveries of Kay *et al*, (2005), providing possible involvement of

eosinophils through secretion of several fibrogenic and growth factors in the repair and remodeling processes in the airways[25]. This model is however unable to explain the observations of Jenkins *et al*,(2003), where severe airway structural changes including thickening of the basement membrane and smooth-muscle hypertrophy, were observed despite long term anti-inflammatory therapy and absence of eosinophilic infiltration in the airways[26].

The discovery of 14-3-3 proteins as an epithelial cell-induced MMP-1 regulatory factor, opens a new door into a potential inflammation independent paradigm for airway remodeling in asthma. On the other hand, possible regulation of this factor by TH_2 cytokines (IL-4 and IL-13) provides a functional link with the inflammatory processes in asthma. In summary, 14-3-3 is an important factor in epithelial-mesenchymal communication which might provide a valuable tool for studying the interrelationships between inflammation and remodeling processes in asthma.

4.2 Future Research

The results and conclusions presented in this thesis provide insight into regulation of MMP-1 expression in lung fibroblast. However several questions still remain.

It is still not clear how 14-3-3 proteins (α/β in the lung and σ in the skin) are secreted? I have excluded the possibility that 14-3-3 is simply released due to cell lysis as lactate dehydrogenase (LDH) was not detected in the media. This implies that 14-3-3 has to be released and/or secreted from live epithelial cells.

Soluble secretory proteins typically contain N-terminal signal peptides that direct them to the translocation apparatus of the endoplasmic reticulum (ER)[27]. Following vesicular

transport from the ER via the Golgi to the cell surface, lumenal proteins are released into the extracellular space by fusion of Golgi-derived secretory vesicles with the plasma membrane[28,29]. As 14-3-3α/β does not harbor any typical amino-terminal ER export signal, the route of its externalization still remains to be determined. Other releasable factors like interleukin-1[30], IL-14 (also known as galectin-1)[31], FGF-2, and endothelial cell growth factor, however, also lack obvious signal peptides and are known to be exported by mechanisms that are operational in the absence of a functional ER/Golgi system[32].

In addition the mechanisms underlying the stimulation of MMP1 expression by 14-3-3α/β are still obscure. Although Lam *et al*, 2005 (unpublished data, see above) have shown that 14-3-3σ stimulates fibroblast MMP-1 levels through the activation of *c-fos* and P38 mitogen activated proteins kinase pathway in dermal fibroblasts. It is yet to be determined whether 14-3-3α/β also upregulates MMP-1 through the same pathway in lung fibroblasts.

Moreover, it is not clear whether extracellular 14-3-3 proteins (α/β in the lung and σ in the skin) bind to a specific receptor on the cell surface of fibroblasts? 14-3-3 proteins have been implicated in a plethora of cellular processes by binding to a consensus recognition motif that has to be phosphorylated at a serine or threonine residue[33]. Several phosphorylation-independent 14-3-3/ligand interactions have, however, been described[34,35]. Since extracellular phosphorylation of 14-3-3 is rather unlikely, the latter mode of ligand binding seems to be more plausible for the interaction of 14-3-3σ and α/β with a putative receptor presented on the cell surface. However, recently Murphy et al (2003) has shown a novel mechanism for extracellular protein- phosphorylation at cell

surface membrane[36]. Continued experimentation is needed to determine whether 14-3-3 proteins also can be phosphorylated extracellularly. Another unanswered question is whether the secreted 14-3-3 protein exists as a monomer or forms a dimer, which is necessary for interaction with phosphorylated 14-3-3-consensus motifs[37].

With regards to specific role of 14-3-3α/β and/or other isoforms of 14-3-3 in lung, further experiments with asthmatic versus non-asthmatic epithelial cells and fibroblasts *in vitro* are required to quantify and compare the amount of releasable 14-3-3 protein in asthmatic as opposed to normal epithelial cells. Moreover, with 14-3-3 proteins being abundant intracellular proteins in all eukaryotic cells[38], it is interesting to study whether in addition to epithelial cells, inflammatory cells of asthmatic airways, (i.e, Th2 cells, eosinophils, neutrophils and basophils) are also able to secrete 14-3-3 proteins.

The discovery of a new function of 14-3-3 proteins opens an interesting and fertile ground for future research in tissue remodeling.

4.3 Reference List

1. Ghahary, A. *et al.* Keratinocyte-releasable stratifin functions as a potent collagenase-stimulating factor in fibroblasts. *J Invest Dermatol.* **122**, 1188-1197 (2004).

2. Lam, E., Tredget, E. E., Marcoux, Y., Li, Y., & Ghahary, A. Insulin suppresses collagenase stimulatory effect of stratifin in dermal fibroblasts. *Mol.Cell Biochem.* **266**, 167-174 (2004).

3. Pardo, A. & Selman, M. MMP-1: the elder of the family. *Int.J Biochem.Cell Biol.* **37**, 283-288 (2005).

4. Brinckerhoff, C. E. & Matrisian, L. M. Matrix metalloproteinases: a tail of a frog that became a prince. *Nat.Rev.Mol.Cell Biol.* **3**, 207-214 (2002).

5. Mercer, B. A., Kolesnikova, N., Sonett, J., & D'Armiento, J. Extracellular regulated kinase/mitogen activated protein kinase is up-regulated in pulmonary emphysema and mediates matrix metalloproteinase-1 induction by cigarette smoke. *J Biol.Chem.* **279**, 17690-17696 (2004).

6. Cataldo, D. D. *et al.* Matrix metalloproteinases and tissue inhibitors of matrix metalloproteinases mRNA transcripts in the bronchial secretions of asthmatics. *Lab Invest* **84**, 418-424 (2004).

7. Ninomiya, S. *et al.* Matrix metalloproteinase-1 polymorphism of promoter region in sarcoidosis and tuberculosis patients. *Sarcoidosis.Vasc.Diffuse.Lung Dis.* **21**, 19-24 (2004).

8. Su, L. *et al.* Matrix metalloproteinase-1 promoter polymorphism and lung cancer risk. *Cancer Epidemiol.Biomarkers Prev.* **14**, 567-570 (2005).

9. Woessner, J. F., Jr. MMPs and TIMPs--an historical perspective. *Mol.Biotechnol.* **22**, 33-49 (2002).

10. White, L. A., Mitchell, T. I., & Brinckerhoff, C. E. Transforming growth factor beta inhibitory element in the rabbit matrix metalloproteinase-1 (collagenase-1) gene functions as a repressor of constitutive transcription. *Biochim.Biophys.Acta* **1490**, 259-268 (2000).

11. McCawley, L. J. & Matrisian, L. M. Matrix metalloproteinases: they're not just for matrix anymore! *Curr.Opin.Cell Biol.* **13**, 534-540 (2001).

12. Fowlkes, J. L., Suzuki, K., Nagase, H., & Thrailkill, K. M. Proteolysis of insulin-like growth factor binding protein-3 during rat pregnancy: a role for matrix metalloproteinases. *Endocrinology* **135**, 2810-2813 (1994).

13. Fowlkes, J. L., Enghild, J. J., Suzuki, K., & Nagase, H. Matrix metalloproteinases degrade insulin-like growth factor-binding protein-3 in dermal fibroblast cultures. *J Biol.Chem.* **269**, 25742-25746 (1994).

14. Imai, K., Hiramatsu, A., Fukushima, D., Pierschbacher, M. D., & Okada, Y. Degradation of decorin by matrix metalloproteinases: identification of the cleavage sites, kinetic analyses and transforming growth factor-beta1 release. *Biochem.J* **322 (Pt 3)**, 809-814 (1997).

15. Whitelock, J. M., Murdoch, A. D., Iozzo, R. V., & Underwood, P. A. The degradation of human endothelial cell-derived perlecan and release of bound basic fibroblast growth factor by stromelysin, collagenase, plasmin, and heparanases. *J Biol.Chem.* **271**, 10079-10086 (1996).

16. Rajah, R. *et al.* Elevated levels of the IGF-binding protein protease MMP-1 in asthmatic airway smooth muscle. *Am J Respir Cell Mol.Biol.* **20**, 199-208 (1999).

17. Phipps, S. *et al.* The relationship between allergen-induced tissue eosinophilia and markers of repair and remodeling in human atopic skin. *J Immunol.* **169**, 4604-4612 (2002).

18. Uria, J. A., Jimenez, M. G., Balbin, M., Freije, J. M., & Lopez-Otin, C. Differential effects of transforming growth factor-beta on the expression of collagenase-1 and collagenase-3 in human fibroblasts. *J Biol.Chem.* **273**, 9769-9777 (1998).

19. Izuhara, K. [Involvement of IL-4 and IL-13 signals in the pathogenesis of bronchial asthma]. *Arerugi* **54**, 7-11 (2005).

20. Richter, A. *et al.* The contribution of interleukin (IL)-4 and IL-13 to the epithelial-mesenchymal trophic unit in asthma. *Am J Respir Cell Mol.Biol.* **25**, 385-391 (2001).

21. Zhu, Z. *et al.* Pulmonary expression of interleukin-13 causes inflammation, mucus hypersecretion, subepithelial fibrosis, physiologic abnormalities, and eotaxin production. *J Clin.Invest* **103**, 779-788 (1999).

22. Rankin, J. A. *et al.* Phenotypic and physiologic characterization of transgenic mice expressing interleukin 4 in the lung: lymphocytic and eosinophilic inflammation without airway hyperreactivity. *Proc.Natl.Acad.Sci U.S.A* **93**, 7821-7825 (1996).

23. Dougherty, M. K. & Morrison, D. K. Unlocking the code of 14-3-3. *J Cell Sci* **117**, 1875-1884 (2004).

24. Blennow, K., Lind, B., Andersson, E., & Andreasen, N. [CSF-analyses in clinical diagnosis of Creutzfeldt-Jakob disease. A literature review and three cases from routine clinical practice]. *Lakartidningen* **98**, 2446-2451 (2001).

25. Kay, A. B. The role of eosinophils in the pathogenesis of asthma. *Trends Mol.Med* **11**, 148-152 (2005).

26. Jenkins, H. A. *et al.* Histopathology of severe childhood asthma: a case series. *Chest* **124**, 32-41 (2003).

27. Walter, P., Gilmore, R., & Blobel, G. Protein translocation across the endoplasmic reticulum. *Cell* **38**, 5-8 (1984).

28. Palade, G. Intracellular aspects of the process of protein synthesis. *Science* **189**, 347-358 (1975).

29. Mellman, I. & Warren, G. The road taken: past and future foundations of membrane traffic. *Cell* **100**, 99-112 (2000).

30. Rubartelli, A., Cozzolino, F., Talio, M., & Sitia, R. A novel secretory pathway for interleukin-1 beta, a protein lacking a signal sequence. *EMBO J* **9**, 1503-1510 (1990).

31. Cooper, D. N. & Barondes, S. H. Evidence for export of a muscle lectin from cytosol to extracellular matrix and for a novel secretory mechanism. *J Cell Biol.* **110**, 1681-1691 (1990).

32. Nickel, W. The mystery of nonclassical protein secretion. A current view on cargo proteins and potential export routes. *Eur.J Biochem.* **270**, 2109-2119 (2003).

33. Yaffe, M. B. How do 14-3-3 proteins work?-- Gatekeeper phosphorylation and the molecular anvil hypothesis. *FEBS Lett.* **513**, 53-57 (2002).

34. Sribar, J. *et al.* The neurotoxic phospholipase A2 associates, through a non-phosphorylated binding motif, with 14-3-3 protein gamma and epsilon isoforms. *Biochem.Biophys.Res.Commun.* **302**, 691-696 (2003).

35. Hallberg, B. Exoenzyme S binds its cofactor 14-3-3 through a non-phosphorylated motif. *Biochem.Soc.Trans.* **30**, 401-405 (2002).

36. Mishra, S. & Murphy, L. J. Phosphorylation of insulin-like growth factor (IGF) binding protein-3 by breast cancer cell membranes enhances IGF-I binding. *Endocrinology* **144**, 4042-4050 (2003).

37. Aitken, A. Functional specificity in 14-3-3 isoform interactions through dimer formation and phosphorylation. Chromosome location of mammalian isoforms and variants. *Plant Mol.Biol.* **50**, 993-1010 (2002).

38. Fu, H., Subramanian, R. R., & Masters, S. C. 14-3-3 proteins: structure, function, and regulation. *Annu.Rev.Pharmacol.Toxicol.* **40**, 617-647 (2000).